Immunomodulation by RNA

Dissertation

zur

Erlangung der naturwissenschaftlichen Doktorwürde
(Dr. sc. nat.)

vorgelegt der

Mathematisch-naturwissenschaftlichen Fakultät

der

Universität Zürich

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Zürich, 2018

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1. Summary

Recombinant *in vitro* transcribed messenger RNA (ivt mRNA) is a versatile and safe vehicle for gene therapy and for vaccination (Pascolo, 2008). It is immunostimulating when composed of unmodified nucleotides (stimulation of innate receptors such as Toll Like Receptors (TLR)-3,7 and -8) or non immunostimulating when containing natural modified nucleotides (e.g. m6A, Ψ) (Kariko et al., 2005; Kariko et al., 2008). Even though naked ivt mRNA coding tumor antigens have been used in clinical studies to vaccinate cancer patients against tumors (Rittig et al., 2011; Sahin et al., 2017a; Weide et al., 2008), improvements of mRNA-based vaccines are being made by encapsulation of mRNA in particles (lipoplexes or polyplexes) (Demoulins et al., 2016; Kranz et al., 2016b). In parallel, gene-therapy approaches in development also use encapsulation of non-immunostimulating ivt mRNA in targeted particles (Stadler et al., 2017; Thran et al., 2017). In order to further improve ivt mRNA-based therapies we studied the role of natural modifications on RNA and optimized functionality of ivt mRNA.

In the first aspect we studied immunostimulating capacities of natural RNA extracted from several organisms to stimulate the human innate immune system and could evidence that a drug, namely Pentostatin can be used to "demodify" endogenous RNA enhancing its triggering of innate immunity (induction of interferon-alpha).

In the second aspect, we optimized the primary structure of ivt mRNA namely by modifying the 5′ untranslated region and also validated formulations that allow targeted expression *in vivo*.

Those fundamental and translational studies allow better understanding of RNA biology and give new options for therapies, including anti-cancer treatments. Combining standard of care and immunomodulation is foreseen as a way towards more efficacious and safer treatments. The immunological fitness of treated patients is thereby a key to treatment's success. Assessing this parameter in patients recruited in a clinical study led us to identify interferon gamma receptor 1, TLR2 and β2-microglobulin as potential surrogate markers for response to chemotherapy. We foresee that a better understanding of RNA biology, an optimized formulation of ivt mRNA and a better characterization of patient's immune system will help in designing personalized efficacious treatments to cure cancers.

2. Zusammenfassung

Die rekombinante in-vitro transkribierte Boten-RNA (ivt mRNA) ist ein vielfältiges und sicheres Vehikel für Gentherapien Impfungen (Pascolo, 2008). Sie ist immunstimulierend, wenn sie aus unmodifizierten Nukleotiden zusammengesetzt ist (Stimulierung von angeborenen Immunrezeptoren wie die Toll-Like Rezeptoren (TLR)-3, 7 und -8). Sie ist nicht immunstimulierend, wenn sie natürlich-modifizierte Nukleotide enthält (z. B. m6A, Ψ) (Kariko et al., 2005; Kariko et al., 2008). Wenngleich auch schon in klinischen Studien nackte, für Tumorantigene kodierende ivt mRNAs zur Impfung von Krebspatienten verwendet worden sind (Rittig et al., 2011; Sahin et al., 2017a; Weide et al., 2008), werden aktuell, durch eine Einkapselung von mRNA in Partikel (Lipoplexe oder Polyplexe), Verbesserungen von mRNA-Impfstoffen erzielt (Demoulins et al., 2016; Kranz et al., 2016b). Die Einkapselung von nicht-immunstimulierender ivt mRNA in zielgerichtete Partikel wird parallell auch verwendet in sich in der Entwicklung befindenden Gentherapieansätzen (Stadler et al., 2017; Thran et al., 2017).

Um die ivt mRNA-basierte Therapie weiter zu verbessern, haben wir den Effekt von natürlichen Modifikationen auf die RNA untersucht und haben dadurch versucht, die Funktionalitat der ivt mRNA zu optimieren.

Im einem ersten Ansatz haben wir die Fähigkeit von natürlicher, aus verschiedensten Organismen extrahierter RNA, das menschliche angeborene Immunsystem zu stimulieren, untersucht. Dabei konnten wir zeigen, dass der Arzneistoff Pentostatin zur «Entmodifizierung» von endogener RNA verwendet werden kann, welches ihre Stimulierung der angeborenen Immunität noch weiter verstärkt (Induktion von Interferon-alpha).

In einem zweiten Ansatz haben wir die Primärstruktur der ivt mRNA durch Modifikation des 5′ untranslatierten Bereichs optimiert. Weiter haben wir unterschiedliche Formulierungen bezüglich zielgerichteter Expression in-vivo validiert.

Unsere Grundlagen- und translationale Studien erlauben ein besseres Verständnis der RNA-Biologie und ergeben neue Therapiemöglichkeiten wie z.B. in der Tumorbehandlung. Die Kombination von aktuellem Behandlungsstandard und der Immunmodulation ist ein Weg in Richtung effizientere und besser verträgliche Therapien. Dabei ist die immunologische Fitness der behandelten Patienten der Schlüssel zum Therapieerfolg. Patienten in einer klinischen Studie, welche zur Evaluierung dieser Parameter durchgeführt wurde, zeigten den Interferon gamma-Rezeptor 1, TLR2 und β2-Mikroglobulin als potenzielle Surrogatmarker für das Ansprechen auf

Chemotherapien. Ein besseres Verständnis der RNA-Biologie, eine optimierte Formulierung der ivt mRNA sowie eine bessere Charakterisierung des Immunsystems der Patienten wird bei der Planung von personalisierten, wirksamen Therapien zur Heilung von Krebs in Zukunft von grossem Nutzen sein.

3. Acknowledgements

It is not the strongest of the species that survives, nor the most intelligent, but the most adaptable to change.

Charles Darwin

Five years ago, my Ph.D. journey began in Switzerland. Leaving home to live abroad for the first time wasn't an easy task but it also came as a spontaneous decision at the end of my Master studies. The language barrier, cultural differences, and cold climate were some of the expected challenges that came my way at the beginning of my studies. Luckily, in all my working environments I was surrounded by people willing to listen, share, help, criticize and support me besides their engagement at work. Therefore, this thesis wouldn't be complete without adding your names and dedicating some lines as a small 'thank you' for all experiences in the last five years.

Although I've started my Ph.D. in a different field and department, almost everyone that I've had the pleasure to meet during those first ten months stayed to support me until the present day.

Here I'd like to thank Dagmara, Elena, and Alex, my first friends in Zurich. Since our meeting at the interviews of the MIM program, we have always tried to stick together. These girls never missed to surprise me for my birthday and gave their best to enrich my social life. I have to thank particularly Dagmara for introducing me to the unique Polish "society" that would find any reason to celebrate and bring people together from all countries and cultures. From this Polish group, Marta, when I met her, was the most experienced Ph.D. student, knowing all the answers to my questions and never complaining about anything. After finishing her Ph.D., she continued being my special guide, unselfishly sharing her expertise and knowledge in neuroscience until the present day.

Katharina, Petra, Mira, and Ivana were my major support during my first Ph.D., helping me make critical decisions, supervising me in my work and reminding me how important is to enjoy the projects we work on. Fabienne, Eugenia, Susan, Silvia, Brenda, Jay, Sasha, Kacper, Wolfgang, Osi, and Ivan were some of the people I had the pleasure to work and share work providing a friendly environment in the department of neurology/clinical immunology.

Ever since I moved to Dermatology, many people came into my life. I will always be grateful to Assen, Anna, and Guergana for being my first guides in Dermatology and helping me integrate into a new department.

People from my office, F2, spiced up my everyday's life. From small talks to big discussions, that has been the place where a little bit of everything was rolled into one space. From all the people that passed through my office, Ásdís was the one starting around the same time as I did and staying until the present day. Strongly motivated and organized in her daily tasks, she never seemed to miss one, training, work, competing, presenting, studying and much more. She managed all that, making us proud to have a high-class athlete and a joyful friend in our lab/office. Now that she is approaching the very end of her Ph.D. I want to wish her best of luck for her future career and I hope she will continue surprising us with her competitive spirit and give us all another chance to support her in the next Olympic games.

Somebody that I really enjoy working and sharing my day is my friend Marilena. It might be we are both southern souls, but somehow between us, everything came and stayed spontaneous. Ever since she met me, Marilena was trying to be protective in my regard, trying to bring up my confidence and push me through my daily ups and downs. Her beauty lies in her endless sense of humor shaped by her feeling for others. Besides keeping the atmosphere and cheering not only me but all of us in the lab/office she'll always be the one without a working day wouldn't be the same and a trip wouldn't be a joyride. May we continue sharing our days wherever life might bring us.

My other spirit neighbor Desi, is our one and only star of F2. The day seems to start only when she walks into the room and loudly shares a good morning with all of us. From the first day, she was trying to remind me not be so serious about temporary things. She would try to cheer me up with her special sense of humor and remind me how I should enjoy my free time while I'm young. No matter how hard her days might be, the smile on her face never goes away giving us all a daily dose of positiveness we don't want to miss.

Egle, Yun Tsan, Joelle, Valerie, Bettina, and Regina are some of the people that were and are part of F2 team and I need to thank for daily contributing to a pleasant and supportive atmosphere. I'd like to especially thank Regina majorly helping with the writing of the German summary in this thesis.

I want to thank our biobank team: Dani, Mirka, Alice, Aga and Tabsi for their availability, keeping the good lab atmosphere and trying to teach me German, particularly Tabsi who invested her free time before work for over a year to teach me German basics. All together you made the lab a working-friendly environment for all of us. People from F floor: Ying, Kacper, Stas, Marta, Jenny, Marieke, Gerhard, Gabriele, Mark, Taka, Barbara, Paulina, Theodora, Franziska, Phil, Sandra,

Johannes, Monika, Dietmar, Emanuel, Jorge, Jessy, Nada were always very helpful and willing to share their expertise and experiences from different fields.

From the people of F floor, I have to say I had the pleasure to meet Serena, my daily companion wherever I am, a great listener and advisor and as I like to call her my younger sister. She is one of those people that never complains, works hards and lives her life fully until her legs can't hold her anymore. Her spirit to explore and challenge herself in all possible tasks are only a few things I admire about her. I hope no matter where we end up after our studies will keep being there for each other in all our life journeys. Ti voglio troppo bene e grazie di tutto!

Besides having a younger sister, I was lucky enough to expand my Zurich family with Jelena and Sonja. Jelena, my "older sister", the voice of sanity and scientific inspirer. Whether is it work or friends issues she tackles, she never discloses how much she takes over her shoulders. I see her as a diligent worker always coming up with new ideas that keep pushing her daily goals further and further. Many times, I wonder from where her passion stems from and how she persists in her daily, weekly, monthly schedules. Now she is about to embark on a new challenging project, on another continent, in a different culture, where coffee breaks and lunches in Bistro Sud, or the after work walks to HB and home visits won't be like ours… However, what I'm sure is that she won't back down on her goals no matter the obstacles. I know she will succeed in any path she decides to take and I'll keep on being by her side like she was always for me in all these years.

Sonja is a very special friend of mine. The resourceful and multitasking Zurich wonder woman shuffling between work, kids, friends, social events, tour guiding, globe-trotting and problem-solving. Extraordinary in many ways and yet one of us. She was the one always "one call away" in any good or bad moments, taking care that I would never feel lonely or that I miss something. Since she experienced it all and decided to turn her existence into life coaching for all of us I can only be grateful for being part of her world. Her endless source of energy and devotion to her family, friends, and work is something that I've only seen in my mother, that's why Sonja is like a second mother to me without whom these past years and my presence wouldn't be the same.

Some of the people that are not in Zurich but keep on supporting me in our year-lasting friendships: Jovana, Anja, Nives, Alek, Nikola, Nada and Tijana, I want to thank for understanding, helping, supporting, cheering, listening and caring for all my stories. Especially thanks to my friend Jovana, who besides our distance navigated with me through this Ph.D., trying to guide me and take out the best parts of me on every occasion.

I want to thank my thesis committee members for all the fruitful discussions and suggestions during the committee meetings helping and supporting the progress of my Ph.D. projects. All these projects wouldn't be possible without my supervisor Dr. Steve Pascolo. He was the one giving me the chance to do my Ph.D. studies on challenging and exciting projects that thought me and still are teaching me new things. Thanks to him I had the chance to explore the world in our working trips, expand my scientific and cultural horizons. Working with him is a great pleasure since he keeps on surprising me with new knowledge and discoveries. Besides my committee members, I have to thank Prof. Pål Johansen for his availability, spirit, altruism and scientific discussing in our lab meetings and daily work.

The most important thanks, I left for this very end and is dedicated to my family. My parents and my brother are my everything. No heart pouring words can explain how much I'm proud to be your daughter and sister. If it wasn't for you I would have never had the chance to pursue this path and to persist in it.

Full of understanding for my ambitions and hopes, I want to thank you with our familiar sayings:

"Tvrd je orah voćka čudnovata, ne slomi ga, al zube polomi!"

Petar Petrović Njegoš

"Onaj koji ne iznosi nježni cvijet svoje duše na vjetrove iskušenja, pa makar ga i cijela spasio i prenio do kraja, tome je kao da ga nikad nije ni imao."

Ivo Andrić

4. Introduction

4.1. RNA modifications

- Presence and diversity of RNA modifications

Similarly to genomic DNA carrying and being regulated by natural nucleotide modifications, RNA molecules from Eukarya, Bacteria, or Archaea harbor modifications and some of them have been known for decades (Dunn, 1961). To the present day over hundred RNA modifications have been identified (Cantara et al., 2011; Machnicka et al., 2013) and listed in dedicated databases (http://mods.rna.albany.edu/; http://modomics.genesilico.pl).

Diverse and abundant modifications are introduced in most cellular RNAs during RNA maturations steps. These modifications are made on the canonical A, C, G, and U residues and their formation is catalyzed by numerous specific enzymes - RNA modification enzymes or RNA-protein complexes (RNPs). Ribonucleotide derivatives can bear single or multiple modifications on:

- the purine/pyrimidine ring where every single position is a potential target for methylation, hydroxymethylation, deamination, transglycosylation, acetylation, reduction, thiolation, oxidation, ribosylation, formylation, isomerization, selenation, or addition of multiple groups; and/or
- the nucleotide's ribose can have the 2′-hydroxyl group methylated or ribosylated with an adenosine-5′-phosphate group (Figure 1).

These naturally occurring modified nucleosides play various structural and functional roles in different types of RNAs: transfer (tRNA) and ribosomal (rRNA), but also in messenger mRNA and small nuclear (snRNAs), micro (miRNA) and long non-coding RNA (lncRNA). The most widespread RNA modifications detected in RNA populations are base or ribose methylations,

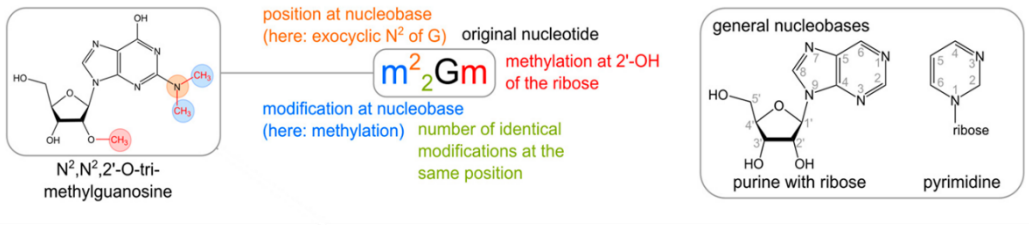

Figure 1. Abbreviations used to define position and type (e.g. methylation) of RNA modifications. Adapted from (Lorenz et al., 2017).

deamination of adenosine to inosine and isomerization of uridine into pseudouridine (Ψ). Special emphasis on these modifications will be devoted in the following parts.

4.1.1. Adenosine related modifications

4.1.1.1. *N6-methyladenosine (m6A)*

4.1.1.1.1. Discovery of m6A

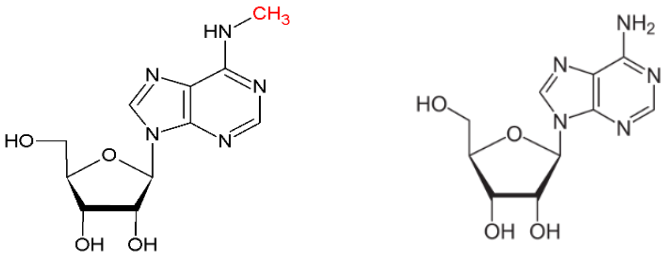

Figure 2. Structure of m6A (left) and adenosine (right). Adapted from URL: http://modomics.genesilico.pl/reactions/A:m6A/

While characterizing the 5′ cap structure of mRNA in mammalian cells researchers discovered the presence of adenosine variants, bearing a methylation on the exocyclic nitrogen and named N6- methyladenosine (m6A) (Figure 2) (Desrosiers et al., 1974).

Ever since the first studies, m6A has been mapped in the RRACH consensus, A being methylated (R=A, G: H=A, C, U), where the first nucleotide next to m6A from the 5′ end is most frequently G. From those studies it was evident that m6A is maintained during processing of the precursor mRNA into mature mRNA (Schibler and Perry, 1977). Follow-up studies confirmed that m6A is introduced in the nucleus and completely retained in the cytoplasmic mRNA (Chen-Kiang et al., 1979). Current estimations classify m6A as the most abundant modification in mRNA occurring on average three sites per mRNA (Rana and Tuck, 1990). Nonetheless, m6A was identified also in rRNA (Maden, 1990), tRNA (Iwanami and Brown, 1968), snRNA (Bringmann and Luhrmann, 1987), miRNA (Alarcón et al., 2015b) and lncRNA (Liu et al., 2013b).

4.1.1.1.2. Detection of m6A

Localizing of m6A in the transcriptome has been hampered by two facts: (i) m6A, similar to A, reverse-transcribes to a thymine (T), and (ii) m6A is not prone to chemical modifications that might facilitate its detection. Two groups in 2012 developed an antibody-based deep sequencing method for the first transcriptome-wide m6A distribution (m6A/MeRIP-seq). In each method, mammalian mRNA is fragmented to 100 nucleotide (nt) sized fragments and immunoprecipitated using an m6A-specific antibody. From immunoprecipitated and input control fragments libraries are made and later on subjected to high-throughput sequencing. These maps have shown that m6A is widely distributed in more than 7,000 mRNA transcripts in human cells, preferably around stop

codons, in 3′ untranslated regions (3′ UTRs) and within long internal exons (Dominissini et al., 2012; Meyer et al., 2012). However, antibody-based sequencing cannot reveal which adenosine residue is modified in a precipitated fragment. With the intention of quantifying the percentage of m6A at a specific site with single-nucleotide resolution new methodologies have been recently developed (Linder et al., 2015; Liu et al., 2013b). One of them, site-specific cleavage and radioactive labelling followed by ligation-assisted extraction and thin layer chromatography (SCARLET), combines: RNase H site-specific cleavage, splinted ligation (RNA bridging to DNA oligonucleotide), ribonuclease digestion, and thin layer chromatography (TLC), to directly determine m6A modification status at any mRNA/lncRNA site from the total RNA sample without the need of purifying a specific RNA (Liu et al., 2013b). The SCARLET method was defined as time-consuming, not feasible for high-throughput applications; therefore photo-crosslinking-assisted m6A-sequencing (PA-m6A-seq) was developed. This method shows improved accuracy of the methylation site assignment and provides a high-resolution (~ 23 nt) transcriptome-wide mammalian m6A map (Chen, 2015). Recently, m6A individual-nucleotide-resolution cross-linking and immunoprecipitation (miCLIP) has been developed with exploiting m6A specific antibodies and specific mutational signatures at m6A residues after ultraviolet light–induced antibody-RNA cross-linking and reverse transcription. This mapped m6A and another modification N6, 2′-O-dimethyladenosine (m6Am) at single-nucleotide resolution in human and mouse mRNA. In addition, m6A has been detected in small nucleolar RNAs (snoRNAs). When validating m6A positive and negative sites with miCLIP, authors found higher specificity and sensitivity in comparison to SCARLET methodology (Linder et al., 2015).

Based on profiled m6A RNA methylomes across many eukaryotes, including human, mouse (Dominissini et al., 2012), yeast (Schwartz et al., 2013), and plant (Li et al., 2014; Luo et al., 2014), it can be said that mRNA methylation is conserved and dynamic. Consistent with previous studies m6A confines to the consensus RRACH sequence and its distribution varies in the transcriptome, favoring sites near stop codons, in 3′ UTRs, and within long internal exons. Additionally, m6A-modified genes are quite well conserved between human and mouse embryonic stem cells (ESCs) and somatic cells. However, distinct m6A patterns can also be detected among different species or cells at different developmental stages (Batista et al., 2014; Geula et al., 2015; Meyer et al., 2012; Schwartz et al., 2013). Some m6A signatures are tissue-specific (Meyer et al., 2012) and altered

in response to different stimuli (Bodi et al., 2012) pointing to the potential role of m6A in regulating diverse cellular processes.

4.1.1.1.3. m6A enzymes

M6A modifications had long been considered static and invariable. Although the enzymatic complex responsible for introducing m6A is known since early 90s (Bokar et al., 1994), enzymes removing it were only recently identified (Jia et al., 2012; Zheng et al., 2013a) proving m6A a reversible post-transcriptional modification (Figure 3).

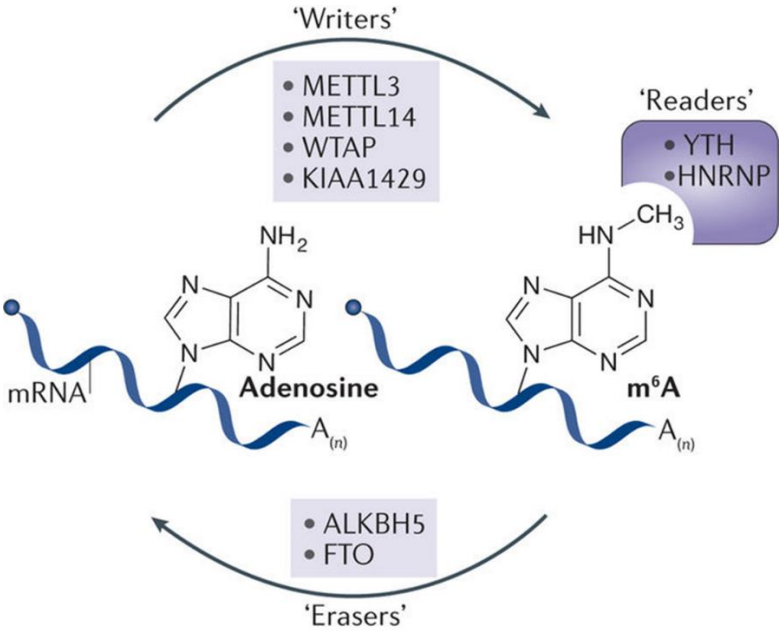

Figure 3. Illustration of enzymatic machineries writing, reading and erasing dynamic m6A. Adapted from (Zhao et al., 2016).

4.1.1.1.4. Writers

'Writers' is a term given for enzymes that are part of the methyltransferase complex introducing m6A. Components of this complex are methyltransferase-like 3 (METTL3) (Bokar et al., 1997), METTL14 (Liu et al., 2014), Wilms tumor 1-associated protein (WTAP) (Ping et al., 2014) and KIAA1429 (Schwartz et al., 2014b).

- METTL3

METTL3 also known as methyltransferase-A70 (MT-A70), is a 70 kDa protein that was the first identified component of the methyltransferase complex isolated from Henrietta Lacks (HeLa) cell nuclear extracts. In this study it has been shown that the knockdown of METTL3 in HeLa cells resulted in a ~30% decrease of the total m6A level (Bokar et al., 1994), while in human hepatoma

G2 (HepG2) cells the same knockdown induced apoptosis, possibly due to upregulation of p53 pathway (Dominissini et al., 2012). METTL3 contains two subunits: consensus methylation motif I (CM I) that binds the methyl-donor for methylation reaction, and CM II that contains catalytic residues for methylation activity (Bokar et al., 1997). METTL3 is highly conserved in eukaryotes, and homologues in yeast (Clancy et al., 2002), plant (Bodi et al., 2012) and *D. melanogaster* have also been identified (Bujnicki et al., 2002). It has been detected in nucleus and cytoplasm (Chen et al., 2015), suggesting that mRNA methylation could be taking place in both compartments, which is consistent with early studies showing that cytosolic extracts also possessed RNA methyltransferase activity (Harper et al., 1990).

- METTL14

Phylogenetic analysis of MT-A70 family indicated 43% of homology of METTL14 with METTL3 (Bujnicki et al., 2002). METTL14 knockdowns in HeLa and Human embryonic kidney 293 FT (HEK293FT) cells decreased m6A levels in mRNA. METTL14 forms a stable heterodimer with METTL3 and together this complex shows higher methylation activity of known m6A consensus sequence in comparison to separate components. METTL3 and METTL14 co-localize in nuclear speckles, and the heterodimer forms the core of the mammalian m6A methyltransferase complex (Liu et al., 2014). Until recently, it has been believed that both METTL3 and METTL14 could potentially catalyze m6A methylation in the METTL3/METTL14 complex. However, structural analysis showed S-adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH) in the catalytic cavity of METTL3, proving that METTL3 is the catalytic subunit of METTL3-METTL14 complex. The role of METTL14 is restricted to stabilization of the complex and recognition of RNA substrates (Wang et al., 2016b).

- WTAP

Wilms tumour 1-associated protein (WTAP) was initially identified as a protein interacting with a WT1 (Wilms' tumour suppressor gene) protein, localized in the nucleus and co-localizing in the nuclear speckles with mRNA splicing factors (Little et al., 2000). However, studies in *Arabidopsis thaliana* (*A. thaliana*) and yeast revealed that homologs of human WTAP, At FIP37 and Mum2 respectively, interact with the *A. thaliana* ortholog of the yeast and human METTL3 (MT-A70), suggesting its role in m6A methylation reactions (Agarwala et al., 2012; Zhong et al., 2008). Moreover, WTAP has been shown to interact with the METTL3–METTL14 complex (Ping et al., 2014). Knockdown experiments of WTAP reduced m6A levels in mRNA, although WTAP alone

did not show any methyltransferase activity *in vitro*. These findings assigned WTAP a regulatory role in the methyltransferase complex. A recent study identified WTAP-dependent and -independent m6A modification sites, where WTAP-dependent sites are located at internal positions in transcripts, topologically static across diverse human and mouse mRNA, while WTAP-independent sites form at the first transcribed base as part of the cap structure. Also in the same study, KIAA1429, an additional candidate of m6A methyltransferase complex has been validated in knockdown experiments as a requirement for complete methylation in mammals (Schwartz et al.). WTAP-null mice exhibit embryonic lethality.

4.1.1.1.5. Erasers

Two m6A erasers which are demethylases, have been reported: fat mass and obesity-associated protein (FTO) and alkB homologue 5 (ALKBH5).

- FTO

Fatso gene coding for fat mass and obesity-associated protein (FTO) was identified from the fused toes (FT) mouse mutation, where mice bearing this heterozygous mutation showed a fused-toe phenotype (Peters et al., 1999). FTO is a member of Alkb protein family (DNA/RNA repair enzymes (Gerken et al., 2007), initially identified to demethylate 3-methylthymine and 3-methyluracil in synthetic single-stranded DNA (ssDNA) and single-stranded RNA (ssRNA) (Jia et al., 2008), and later shown to be the first identified RNA demethylase catalyzing oxidation of m6A residues in mRNA. *In vitro* studies of FTO knockdown in human cells induced increased amounts of m6A, while overexpression experiments resulted in decreased amounts of m6A in mRNA. Moreover, FTO has been shown to localize in the area of nuclear speckles. These findings characterized m6A as a reversible and dynamic modification (Jia et al., 2012).

Oxidation of m6A by FTO is dependent on Fe(II)- and α-ketoglutarate producing N(6)-hydroxymethyladenosine (hm6A) as an intermediate modification, and N(6)-formyladenosine as a further oxidized product (Fu et al., 2013). Impairment of FTO function affects normal growth development of the central nervous system in mutant mice, and it has been associated with obesity. Single nucleotide polymorphisms within *FTO* gene are associated with increased body mass and obesity in humans (Dina et al., 2007). FTO deficiency in mice caused growth retardation (Fischer et al., 2009). Similarly in humans, loss-of-function mutations in FTO causes malformations, severe growth retardation resulting in premature death (Boissel et al., 2009).

- ALKBH5

Similarly to FTO, alkylation repair homologue protein 5 (ALKBH5) belongs to the iron- and 2-oxoglutarate (2OG)-dependent family of AlkB oxygenases. Knockdown of this protein in human cell lines yields higher m6A levels in mRNA and accelerates its export from the nucleus to the cytoplasm. In the same experiments, ALKBH5 was shown to colocalize with nuclear speckles and associate with mRNA processing factors possibly affecting splicing reactions. Unlike FTO, ALKBH5 tightly interacts with mRNA and directly removes methyl groups from m6A-methylated adenosine without oxidative demethylation (Zheng et al., 2013a), most likely catalyzing the decomposition of m6A intermediate hm6A to adenosine (Zheng et al., 2013b).

Mice deficient in *alkbh5* exhibit defective spermatogenesis possibly resulting from altered expression of spermatogenesis-related genes and high levels of expression of ALKBH5 in mouse testes (Zheng et al., 2013a).

The specificity of FTO and ALKBH5 for nucleotides in target transcripts remains to be elucidated. Current studies suspect that the diversity in structural features in nucleotide binding sites and catalytic domains define which m6A residue on a target sequence undergoes demethylation (Aik et al., 2014; Xu et al., 2014a; Yang et al., 2016).

4.1.1.1.6. Readers

Investigated biological roles of m6A are defined through protein-RNA interactions. Occurrence of m6A in RNA destabilizes RNA duplexes thus promoting binding of RNA-binding proteins to single-stranded transcripts or preventing double-stranded-binding proteins (Kierzek and Kierzek, 2003; Spitale et al., 2015). Proteins regulated through this reaction are known as indirect readers of m6A. Nonetheless, proteins specifically recognizing and binding m6A rich motifs in the RNA have been identified (Dominissini et al., 2012).

- Direct m6A readers

Specific binders of m6A were detected in pull down assays from human cell lysate (Dominissini et al., 2012). These proteins share an YT521-B homology (YTH) domain and are highly conserved in mammals (Stoilov et al., 2002; Zhang et al., 2010). Thus far five members have been qualified as members of YTH protein family: YTH domain family protein 1 (YTHDF1), YTHDF2, YTHDF3 and YTH domain-containing 2 (YTHDC2) localized in the cytoplasm (Bailey et al., 2017; Dominissini et al., 2012; Wang et al., 2014), and YTHDC1 found both in cytoplasm and

nucleus (Berglund et al., 2008; Xu et al., 2014b). First studies have shown higher binding affinity of YTHDF1-3 to methylated probe compared to unmethylated one (Dominissini et al., 2012; Wang et al., 2014). YTH domain contains a hydrophobic socket proven to accommodate methyl group of m6A (Theler et al., 2014). It has been demonstrated that cytoplasmic readers YTHDF1 and YTHDF2 modulate translation and mediate degradation of m6A modified mRNA, respectively (Wang et al., 2014; Wang et al., 2015). Recently, YTHDF3 has been shown to promote translation through interacting 40S/60S ribosomal subunits (Li et al., 2017a). Nuclear reader YTHDC1 regulates m6A-dependent mRNA splicing (Xiao et al., 2016) while YTHDC2 probably has an Adenosine triphosphate (ATP)-dependent RNA helicase function (Fanale et al., 2014).

It has been reported that eukaryotic initiation factor 3 (eIF3), a component of 43S translation preinitiation complex, directly binds with 5′ UTR m6A of mRNA and mediates cap-independent translation particularly in condition of cellular stress (Meyer et al., 2015). Heterogeneous nuclear ribonucleoprotein A2/B1 (hnRNPA2B1) has also been shown to selectively recognize methylated pri-microRNA in order to promote micro-RNA maturation (Alarcón et al., 2015a; Alarcón et al., 2015b).

- Indirect readers

Presence of m6A weakens base pairing and alters local RNA structure (Roost et al., 2015). Such structural changes are referred as m6A switch. M6A switch exposes RNA-binding motifs facilitating the binding of hnRNPC and hnRNPG, proteins identified to ultimately affect alternative splicing of transcripts (Liu et al., 2015; Liu et al., 2017).

4.1.1.1.7. Role of m6A in RNA function and fate

M6A is the most common internal modification in mRNA and long noncoding RNA with significant conservation of m6A modified genes between mouse and human in somatic cell types (Dominissini et al., 2012; Meyer et al., 2012). Studies about evolutionary conservation of m6A methylomes in human embryonic stem cell (hESC) and mouse embryonic stem cell (mESC) identified at the gene level, 69.4% (3,609 of 5,204) of hESC genes are also m6A modified in the orthologous mouse gene, with 46 % of the m6A peak sites in common. This significant overlap at the gene level implied a major role of m6A modification during development in both species (Batista et al., 2014).

Several reports suggested m6A presence in transcripts mediates transition toward differentiation *in vivo*. M6A marking of transcripts in "naïve" ESC and "primed" epiblast stem cells (EpiSCs) facilitates removal of undesired transcripts and allows expression of new gene networks required for proper lineage priming and cell differentiation. METTL3 targets majority of the core pluripotent factors and developmental regulators in hESC and mESC. Knockout of METTL3 in mouse "primed" EpiSCs and "naïve" ESCs led to a reduction of m6A in mRNAs and increased stability of pluripotent mRNA transcripts. Subsequently, these stem cells undergo aberrant and limited potential differentiation at later implantation stages, resulting in early lethality of embryo. Thus, ablation of METTL3 potentiates the already high pluripotency genes in the naive condition to create a hypernaive pluripotent state, but produces the dominating developmental factors in the primed state and counterbalances towards differentiation (Geula et al., 2015).

In a separate study, METTL3 silencing delays the nuclear export of mature mRNAs of clock genes Aryl hydrocarbon receptor nuclear translocator like (*Arntl*) and Period circadian regulator 2 (*Per2*), which connect m6A to the pace of the circadian cycle and the clock speed and stability (Fustin et al., 2013).

- Dosage compensation

M6A is involved in various aspects of sexual development in *Saccharomyces cerevisiae (S. cerevisiae)*, *Drosophila melanogaster (D.melanogaster)* and mammals. Methylation of mRNA in yeast is restricted to meiosis induced by starvation or following prolonged rapamycin treatment. There m6A marking probably promotes translation of specific transcripts (Agarwala et al., 2012; Bodi et al., 2015). In *D.melanogaster* females presence of m6A promotes splicing of Sex lethal (Sxl) thereby enhancing its expression that ultimately inhibits translation of Male-specific lethal-2 (Msl-2) required for transcription from the single male X chromosome (Haussmann et al., 2016). Mammalian long non-coding RNA X-inactive specific transcript (XIST) is highly methylated and m6A enhances its function through its recognition by YTHDC1. XIST mediates transcriptional silencing of genes on the X chromosome (Patil et al., 2016).

- M6A site-dependent expression of mRNA

Several reports suggest that the presence of m6A affects mRNA fate including processing, nuclear export, stability and translation. Human and mouse methylomes indicate m6A is enriched at 3′-UTRs, around stop codons and also marks long exons (Dominissini et al., 2012; Meyer et al., 2012). First reports defined m6A to promote translation efficiency if it is located in 3′ UTR, open

reading frames and near stop codons. Binding of YTHDF1 to m6A near the stop codon and possible interaction with the translation initiation factor eIF3 is suggested to stimulate translation (Wang et al., 2015). However, a recent report suggests that m6A in both the ORF and the 3′ UTR could mediate accelerated poly(A) removal in the cells through recruitment of YTHDF2 that interacts with the deadenylation carbon catabolite repressor factor 4 - negative regulator of transcription (CCR4–NOT) complex resulting in decay of m6A-containing RNAs (Du et al., 2016). Additionaly, in glioblastoma stem cells (GSCs) it has been shown that m6A presence in 3′ UTR of Forkhead box protein M1 (FOXM1) pre-mRNA blocks its expression through preventing the interaction with Human antigen R (HuR) protein and thereby the export from the nucleus (Zhang et al., 2017).

M6A marking of both exons and introns was shown to promote alternative splicing. Binding ability of Serine and arginine-rich splicing factor-2 (SRSF2) has been found to be dependent on m6A, where m6A modified exons are rather included in the mature mRNA (Zhao et al., 2014). Other studies indicate that modified exons are recognized by YTHDC1 in pre-mRNA and that YTHDC1 promotes exon inclusion through recruiting pre-mRNA splicing factor (SRSF3) while blocking SRSF10 binding to pre-mRNA (Xiao et al., 2016).

On the contrary, m6A found in introns has been identified to prevent exon inclusion in mature mRNA. An extensively studied example is the Sxl pre-mRNA. In *D. melanogaster* females' existence of active Sxl suppresses translation of Msl-2, which transcripts are present in both females and males. Msl-2 in males is responsible for upregulation of transcription from X chromosome to compensate the unequal numbers of X chromosomes between males and females. In Sxl pre-mRNA, m6A is present in introns flanking an alternatively spliced exon that encodes for a premature stop codon in males. This exon is excluded in females through Sxl self-regulation of alternative splicing in Sxl pre-mRNA. Sxl binds on either side of the intron, close to m6A sites and with m6A reader YT521-B fully prevents exon inclusion ultimately resulting in high levels of Sxl expression in females. Thus, m6A is required to direct splicing and provide sufficient Sxl for sex determination and dosage compensation (Haussmann et al., 2016).

Recent reports have provided compelling evidence on how m6A localization in 5′ UTR promotes cap-independent mRNA translation. Under stress conditions, ultraviolet (UV) or heat shock induce preferentially 5′ UTR m6A methylation in stress-inducible mRNA, probably to differentiate nascent transcripts from pre-existing mRNA and facilitate selection of mRNA for translation. The

nuclear YTHDF2 preserves 5′ UTR m6A methylation of stress-induced transcripts by limiting access of FTO and thereby demethylation. In absence of a 5′ end cap structure - a methylated purine (N^7-methylguanosine, m^7G) it is possible that m6A acts as a replacement and thereby promotes translation (Meyer et al., 2015; Zhou et al., 2015).

Additionally, it is probable that there is a preference in polyadenilation sites depending on m6A density to its proximity. Namely, in pre-mRNA with two alternative polyA (APA) sites, when a proximal polyA site is chosen more frequently than a distal site, m6A density both before and after this site was shown to be lower, whereas if the proximal polyA site is used less frequently, the m6A density around this site was shown to be greater (Ke et al., 2015).

4.1.1.2. *1-methyladenosine (m1A)*

4.1.1.2.1. Discovery of m1A

Methylation on the N1 atom of adenosine forms 1-methyladenosine (m1A) (Figure 4) identified in RNA since early 1960s (Dunn, 1961; Hall, 1963). This highly conserved RNA modification was found in non-coding RNA, tRNA and rRNA, and recently in mRNA of several human and murine cell lines and in yeast (Dominissini et al., 2016; Li et al., 2016b; Machnicka et al., 2013).

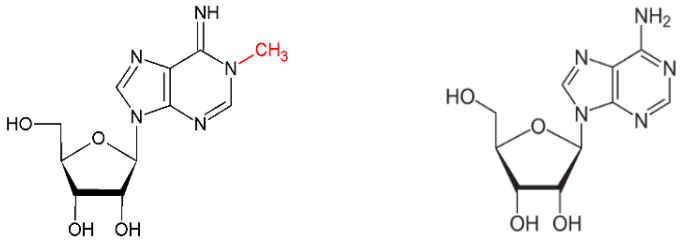

Figure 4. Structure of m1A (left) and adenosine (right). Adapted from URL: http://modomics.genesilico.pl/reactions/A:m1A/

M1A in cytosolic (cyt)tRNA is found on five different positions typically in the tRNA T-loop, with two of them (9 and 58) being in common with mitochondrial (mt)tRNA. Some of these methylations are domain specific: m1A modification in nucleotide position 22 (m1A22) identified only in tRNAs from Bacteria (Matsugi et al., 1992; Menichi et al., 1980) and m1A57 modification found in Archaea, transiently existing as an intermediate leading to 1-methylinosine (m1I) by hydrolytic deamination (Grosjean et al., 1995a). Others have been conserved between domains like m1A58 modification found on (cyt)tRNAs from all three domains of life and further in (mt)tRNAs and m1A9 modification of (cyt)tRNA from Archaea or mammalian (mt)tRNAs (Helm and Attardi, 2004). Rarely occuring m1A14, has so far only been identified in (cyt)tRNA(Phe) from mammals (Sprinzl and Vassilenko, 2005). m1A is found in 18S and 25S rRNA and the responsible machinery for this process has been predominantly investigated in yeast (*S. cerevisiae*) (Meyer et al., 2011; Peifer et al., 2013).

Recently two groups independently performed m1A high-resolution transcriptome-wide mapping detecting m1A in 5′ UTR, coding region and 3′ UTR, with preferential localization in highly structured, GC-rich regions at the 5´ UTR near the start codon (Dominissini et al., 2016; Li et al., 2016b). m1A was found upstream of the first splice site in human mRNA, close to both canonical and non-canonical translation initiation sites (Dominissini et al., 2016). Their work proves m1A, besides m6A, as a reversible and dynamic modification in eukaryotic mRNA. Recently, m1A mapping at single-nucleotide resolution identified GUUCNANNC (A=m1A) as the sequence motif for m1A. According to this mapping m1A is present in a low number of mRNA (Safra et al., 2017).

4.1.1.2.2. Detection of m1A

The N1 of adenosine acts as a hydrogen bond receptor and once bearing a methyl group (m1A) modification becomes positively charged and impairs Watson–Crick base pairing. m1A can cause both reverse transcription stops and read-throughs accompanied by mismatches. This feature has been exploited to sequence m1A at high resolution in mRNA: m1A-seq and m1A-ID-seq developed by Dominissini (et al.) and Li (et al.), respectively (Dominissini et al., 2016; Li et al., 2016b).

Both approaches consist in selection and enrichment by m1A-specific antibody immunoprecipitation. In m1A seq approach a portion of precipitated m1A-containing mRNA fragments are treated with alkaline buffer to chemically rearrange m1A to m6A prior to complementary DNA (cDNA) synthesis exploiting the Dimroth rearrangement reaction. Further, comparison between alkaline buffer treated and untreated samples was performed according to mutational rates, where high mutation rates are found in untreated samples (m1A mutation prone in reverse transcription) and low in the treated ones (m6A low mutation rate in reverse transcription). With this methodology m1A sequencing peaks are at the resolution of 5–15 nucleotides (conserved m1A sites in rRNA can be mapped at the resolution of one nucleotide). In m1A-ID-seq approach, AlkB (an eraser of m1A) from *Escherichia coli (E. coli)* was used to demethylate m1A to regular adenosine. In parallel, since m1A was shown to stop reverse transcription under optimized conditions (low dNTPs), Avian Myeloblastosis Virus (AMV) reverse transcriptase cDNA directed synthesis is performed to confer cDNA truncations near m1A sites. This maps m1A at the resolution of 55 nucleotides by comparing the m1A peak features

between the AlkB untreated and treated samples (Dominissini et al., 2016; Li et al., 2016b). Single-nucleotide resolution m1A transcriptome mapping has been recently developed. The protocol combines steps employed in previous m1A mapping strategies. However, increased reading depth of cDNA libraries obtained from different reverse transcription conditions and inclusion of additional filters in the analytical pipeline detected with high confidence m1A sites (Safra et al., 2017).

4.1.1.2.3. Writers

Two methyltransferase superfamilies: the Rossmann-fold methyltransferase (RFM) and SpoU-TrmD (SPOUT) are responsible for introducing m1A.

SPOUT superfamily enzymes exhibit an unusual alpha/beta fold, resembling Rossman fold, with a trefoil knot. The SPOUT domain binds SAM as a cofactor thus representing the catalytically active subunit of the MTs. Additional nucleic acid binding domains have been identified in SPOUT MTs (Aravind and Koonin, 2001; Tkaczuk et al., 2007). The RFM family is named after the structural motif (Rossmann-fold) known for binding of adenosine-containing cofactors (Motorin and Helm, 2011). It comprises both RNA and DNA MTs containing apart RFM-fold additional domains for substrate recognition (Schubert et al., 2003). Members of these two superfamilies are responsible for m1A modifications at nucleotide position 9, 22, and 58 in tRNA, while MTs of position 14 are unknown. From RFM family tRNA methyltransferase (Trm)K, Trm6/Trm61 and Trm61 alone catalyze methylation of nucleotide position 22 in bacterial tRNA, position 58 in cytosolic and mitochondrial tRNA of Eukaryotes, respectively (Wang et al., 2016a). Trm10 from SPOUT superfamily methylates position 9 in mitochondrial tRNA of Eukaryotes (Motorin and Helm, 2011). The TrmK (m1A22 MT) is conserved in Bacteria, having orthologues in Gram-positive and Gram-negative bacteria, but has no identified homologues in Eukaryotes (Roovers et al., 2008). m1A9 in mitochondrial tRNAs is known to stabilize the canonical cloverleaf structure, preventing tRNA folding into a nonfunctional structure (Helm et al., 1998). The tRNA methyltransferase responsible for m1A58 formation is a heterotetramer complex initially identified in *S. cerevisiae* as a two-subunit enzyme consisting of TRM61 (*GCD14*) and TRM6 (*GCD10*) (Anderson et al., 2000). Trm61 functions as the catalytic subunit, but both Trm61 and Trm6 are required for tRNA binding (Ozanick et al., 2007). TrmI is a homotetrameric enzyme found to catalyze m1A58 in bacterial and archaeal tRNA, discovered as a homolog of yeast Trm61 (Roovers

et al., 2004). Trmt61B was reported as a mitochondria-specific methyltransferase responsible for m1A58 formation in human mitochondrial tRNAs (Chujo and Suzuki, 2012). m1A58 forms in the TΨC loop reverse - Hoogsteen base pairing with 5-methyluridine (m5U) at position 54. The methylation positively charges the elbow region of the tRNA (Agris, 1996). This positive charge is believed to stabilize the tertiary structure of tRNAs, e.g. eukaryotic initiator tRNA(Met) (Basavappa and Sigler, 1991).

Nucleolar essential protein 1 (Nep1) is highly conserved in eukaryotes and is essential for 40S ribosomal subunit maturation (Eschrich et al., 2002). Nep1 was identified to catalyze N1-methylation within loop 35 of the 18S rRNA that contains the unique hypermodification of U1191 1-methyl-3-(3-amino-3-carboxypropyl)-pseudouridine (m1acp3Ψ) (Meyer et al., 2011). The ribosomal RNA processing protein ribosomal RNA processing protein 8 (Rrp8) is known to interact with Nep1 and catalysing N-1-methylation of adenine at position 645 in helix 25.1 of *S. cerevisiae* (Peifer et al., 2013). Base methyltransferase of 25S rRNA (BMT2) was reported to be responsible for m1A2142 base modification of helix 65 in 25S rRNA in the same model organism (Sharma et al., 2013). Specific m1A-MTs acting on mRNA are still under investigation.

4.1.1.2.4. Erasers

ALKBH3, an AlkB-family DNA/RNA demethylase and homolog of ALKBH5 and FTO, was shown to demethylate m1A in mRNA thus proving m1A as a reversible modification. Specific mRNA m1A peaks were identified in *ALKBH3*$^{-/-}$ HEK293T cells, preferentially located in the 5′ UTR suggesting rather a regulatory role of ALKBH3 than its function as a repair enzyme (Li et al., 2016b). Human ALKBH1 is another member of the AlkB family proteins, recently found to mediate removal of m1A in tRNA (Liu et al., 2016).

4.1.1.2.5. Readers

Readers of m1A have not been identified.

4.1.1.2.6. Role of m1A in RNA function and fate

Most of m1A roles have been investigated through deletion or transient ablation of MTs in specific cells *in vitro* or *in vivo*. Deletion of the MTase responsible for N1-methylation A58 in yeast produces non-viable cells while reduction of this MTase by small interfering (si)RNA-mediated knockdown gave rise to a slow-growth phenotype in human cells (Anderson et al., 2000; Saikia et

al., 2010). This effect seen in yeast might be due to its role in stabilizing and helping maturation of initiator tRNA(Met) from yeast. Both TRM61 and TRM6 have been shown to be essential for this process. Upon gene inactivation of either of these two genes, the initiator tRNA(Met) is 3′-polyadenylated by the topoisomerase I related function 4 protein (Trf4p)/Mtr4p complex and subjected to exosome complex-mediated degradation (Kadaba et al., 2004).

Loss of m1A9 modification by knockdown MTs was shown to be embryonically lethal in *D. melanogaster*, while in HeLa cells the knockdown resulted in reduced mitochondrial respiration, sustaining the role of m1A MTs in cell viability (Lopez Sanchez et al., 2011; Sen et al., 2016). The MTase responsible for tRNA m1A22 in *Streptococcus pneumoniae* was also shown to be essential for bacterium survival (Thanassi et al., 2002).

It has been shown that retroviruses use tRNA bearing m1A58 as primers to initiate reverse transcription. Human immunodeficiency virus-1 (HIV-1) exploits presence of m1A58 in human tRNA(Lys) for efficient and accurate reverse transcription (Auxilien et al., 1999).

Depletion of Nep1 and BMT2 have been shown to affect yeast cell growth (Meyer et al., 2011; Sharma et al., 2013).

M1A presence in mRNA was identified to increase translation thus positively correlating with protein production. Being localized at 5′ UTR and translation initiation sites, Li (et al.) suggested m1A is affecting mRNA folding to promote translation initiation. Under serum starvation conditions and H_2O_2 treatment inducible m1A marking of mRNA was found in the 5′ UTR and near start codon regions, similarly to normal conditions, possibly promoting translation of stress induced mRNA (Li et al., 2016b).

4.1.1.3. *Inosine*

4.1.1.3.1. Discovery of adenosine deaminases acting on RNA

Inosine is an RNA modification resulting from a hydrolytic deamination of adenosine (Figure 5) catalyzed by enzymes of the Adenosine Deaminase Acting on double stranded RNA (ADAR) and Adenosine deaminase acting on tRNA (ADAT) families. First report of ADARs were in *Xenopus laevis* oocytes and embryos, where authors named them as proteins with unwinding activity of double stranded RNA (Bass and Weintraub, 1987, 1988; Wagner et al., 1989). In mammals, first

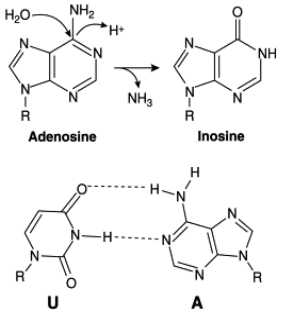

Figure 5. Deamination of adenosine to inosine (A) Base pairing of inosine with cytosine (B). Adapted from (Valente and Nishikura, 2005).

discovered gene from ADAR family was *ADAR1* in humans (Kim et al., 1994b), where soon after also *ADAR2* and *ADAR3* were identified (Chen et al., 2000; Melcher et al., 1996b). Orthologs of these genes are evolutionary conserved in vertebrates and only few genes have been identified in invertebrates. *D. melanogaster* possess one gene of ADAR family *dAdar*, similar to *ADAR2*, while *Caenorhabditis elegans (C. elegans)* has two ADAR genes, *adr-1* and *adr-2*. In plant, fungi and yeast genomes ADAR genes haven't been identified (Jin et al., 2009). Members of ADAT family are responsible for catalyzing deamination of adenosine to inosine on tRNAs in all domains of life. ADATs introduce inosine on three positions on tRNAs: position 34 (Bacteria, Eukaryote), position 37 (Eukaryotes) and position 57 (Archaea). Inosine on position 34 is found in the final modified state while on position 37 and 57 is methylated into m1I37, m1I57 or m1Im57 (Juhling et al., 2009; Machnicka et al., 2013). ADAT family comprises in Eukaryotes: ADAT1 (Tad1), ADAT2 (Tad2) and ADAT3 (Tad3) (Gerber and Keller, 2001).

4.1.1.3.2. Detection of Inosine

A-to-I editing introduces guanosine (inosine) instead of adenosine read in RNA-sequencing by next-generation sequencers (NGS). Thus, this A-to-G conversion could be traced by comparing with the reference genome. However, true RNA editing sites have to be distinguished from genomic variations such as single nucleotide polymorphisms (SNPs). For this purpose, specific analytical pipelines using bioinformatics tools with specific filters have been developed. These filters including general criteria, to prevent inclusion of single nucleotide variations, and specific A-to-I targeted criteria that take into account biological properties of ADARs and localization of putative editing sites such as clustering of editing sites (Peng et al., 2012; Solomon et al., 2013), ***A****rthrobacter* ***lu****teus* (Alu) and non-Alu elements properties (Ramaswami, 2012), tissue-preferred distribution of editing events, RNA folding (Maas et al., 2011). Four bioinformatics tools are available for the detection of RNA editing events from FASTQ files and/or mapped datasets: ExpEdit, GIREMI, RASER, REDItools and two databases to explore RNA editing events DARNED (https://darned.ucc.ie/) (Kiran and Baranov, 2010) and RADAR (http://rnaedit.com/) (Ramaswami and Li, 2014). Most recent bioinformatic tool developed for detection of editing events is RNAEditor. This tool also uses FASTQ files as input and fully automates all steps needed to identify RNA editing events. In RNAEditor a clustering algorithm is implemented to detect regions of highly edited sites referred as 'editing islands'. Compared with a single edited site, an

editing island indicates potential ADAR binding sites, gives higher confidence that the contained editing sites are 'true' editing sites and higher likelihood of biological importance (John et al., 2016).

4.1.1.3.3. Writers

Enzymes of ADAR family possess common functional domains (Figure 6). They have two to three double stranded-RNA binding domains, 65 amino acid long, through which they directly bind to double stranded RNA (dsRNA) (Stefl et al., 2006). On the C-terminus they contain the catalytic deaminase domain forming the catalytic center of an ADAR. According to a proposed catalytical model for deaminase activity, the target A base flipped into the active enzyme site (Macbeth et al., 2005). Some ADARs have a specific structural feature: ADAR1 has two Z-DNA binding domains (Zα and Zβ) (Herbert et al., 1997), for which is believed to play a role in increasing the affinity of binding double stranded RNA, like siRNA; ADAR3 holds an arginine rich domain (R) at its N-terminus that binds single stranded RNA, but its role in ADAR3 is still unknown (Figure 6) (Chen et al., 2000).

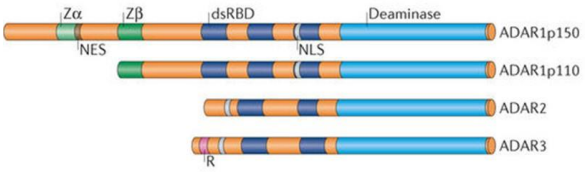

Figure 6. Structural features of ADARs. Adapted from (Nishikura, 2016).

ADAR1 and ADAR2 are functionally active as homodimers, while ADAR3 is a monomer. Almost all editing reactions are attributed to ADAR1 and ADAR2, while ADAR3 doesn't show deaminating activity *in vitro*. Although features of its functional deaminating domain have been conserved, it is believed that the incapability to form a dimer is responsible for the absence of its activity (Chen et al., 2000; Melcher et al., 1996a; Schneider et al., 2014). ADAR1 and ADAR2 are expressed in many tissues, ADAR2 is highly expressed in the brain, while ADAR3 is expressed only in the brain (Chen et al., 2000; Kim et al., 1994b; Melcher et al., 1996b).

ADAR1 gives two protein isoforms: long isoform referred as ADAR1L or ADARp150 and ADAR1S or ADARp110 an isoform having a shorter N-terminus and lacking a Z-binding domain, as a result of generation from different promoters and alternative splicing. ADAR1L is transcribed from a promoter induced by interferon and its expression increased in conditions of cellular stress

or viral infection while ADAR1S mRNA is transcribed from constitutively expressed promoters (George and Samuel, 1999; Patterson and Samuel, 1995).

ADAR1L is mainly localized in the cytoplasm, while ADAR1S localizes in the nucleoplasm and nucleolus, however in conditions of stress (UV irradiation) it translocates in the cytoplasm (Sakurai et al., 2017). ADAR2 is found in the nucleolus (Desterro et al., 2003). The nuclear localization signal (NLS) located in the third double stranded RNA binding domain (dsRBD) drives the nuclear import of both isoforms of ADAR1. In contrast, nuclear export of p150 isoform is mediated by binding of exportin 1 to the nuclear export signal (NES) located within the Zα domain of ADAR1p150, the mechanism of which is different from the export of p110 isoform, requiring exportin 5 (Fritz et al., 2009; Strehblow et al., 2002). ADAR2 N-terminus contains a NLS, which allows ADAR2 to localize into the cell nuclei (Maas and Gommans, 2009). The significance of this differential distribution is unknown. However, it is probable that ADAR1L distribution is owing to the localization of its target molecules in cytoplasm e.g. viral dsRNA or dsRNA that are precursors of mi- and small interfering (si)- RNA.

ADARs are binding to substrates with their dsRBD. Structural features of dsRNA are localized deep in the inner part of the molecule therefore ADARs are not recognizing the specific sequence of targets but rather the editing activity is dependent on the stability of dsRNA substrate (Lehmann and Bass, 1999).

Intermolecular and intramolecular dsRNA structures longer than 20 base pairs (bp) are possible ADAR substrates. Editing destabilizes RNA substrates and probably is ongoing until the enzyme can’t recognize its substrate as dsRNA. On the contrary, in short dsRNA (20 to 30 bp) or long and partially complementary dsRNA possessing wrong base-pairing, single stranded bulges and loops, only few As are specifically edited since deamination of this few As decreases stability of dsRNA substrates below the threshold of recognition of ADAR. Thus, the secondary structure of dsRNA is the one dictating selection of editing places by ADAR (Lehmann and Bass, 1999; Nishikura et al., 1991).

Some editing places are preferential substrates for ADAR1 or ADAR2, which means that the substrate specificity differs between functional forms of ADAR, possibly due to different number and relative distance of dsRBD that can facilitate differing between dsRNA of different structure and stability. Selection of editing places by ADAR1 and ADAR2 might be driven also through functional interactions between ADAR1 and ADAR2 generating heterodimers. Additionally, some

sequence preference has been reported where both ADAR1 and ADAR2 have 5′ neighbor preference (A=U > C=G), and ADAR2 may have 3′ neighbor preference (U=G > C=A) (Lehmann and Bass, 2000). A-to-I editing is found in both coding and non-coding regions but mostly reside in noncoding parts of the human transcriptome, such as introns or 3′ UTRs (Li et al., 2009; Peng et al., 2012; Ramaswami, 2012).

In *Adar1*-/- mice embryonic lethality was evidenced day 12.5 (E12.5) showing ADAR1 is essential for life. These mice show substantial overproduction of interferon and widespread apoptosis (Wang et al., 2000b). Furthermore, in response to inflammation ADAR1 editing is detected in immune organs and lymphocytes. All these findings suggested ADAR1 may have a role in regulating inflammatory and immune responses (Yang et al., 2003). Similarly, human mutations of *ADAR1* gene cause Aicardi-Goutières syndrome, which is characterized by childhood encephalopathy and massive interferon production (Aicardi and Goutieres, 1984). In addition, the deadly phenotype of ADAR1 knockdown mouse has been shown to be reversible with concomitant knockdown of Mitochondrial antiviral signaling protein (MAVS) or Melanoma Differentiation-Associated protein 5 (MDA5) (Liddicoat et al., 2015; Mannion et al., 2014). It has been recently demonstrated that A-to-I editing of endogenous dsRNA by ADAR1 is essential to prevent the activation of the cytosolic dsRNA response by endogenous transcripts (Liddicoat et al., 2015). Moreover, in ADAR1 knockout mice death of embryonic liver hematopoietic cells and liver disintegration were also observed. This could be explained with the finding that ADAR1 plays a role in differentiation of hematopoetic progenitor cells implicating ADAR1 is important in hematopoiesis and organ development (XuFeng et al., 2009).

A large number of ADAR1 mutations (>130) are also associated with dyschromatosis symmetrica hereditaria (DSH), an autosomal-dominant disorder mainly found in Asian individuals and is characterized by altered skin pigmentation (Suzuki et al., 2007).

Mice deficient for ADAR2 die within 3 weeks after birth and are prone to seizures. ADAR2 is known to edit critical position of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor subunit glutamate R (GluR)-B pre-mRNA in the brain, encoding glutamate receptor subunit 2 (Gria2). ADAR2 deficient mice can be rescued when an unedited transcript is substituted with an edited one, suggesting Gria2 as the major target of ADAR2 (Higuchi et al., 2000; Higuchi et al., 1993). RNA editing dysfunction are related also to central nervous system diseases. Decrease in editing activity in GluR-B is responsible for motorneuron death in sporadic

cases of amyotrophic lateral sclerosis, also it probably plays a role in epilepsy. Imbalanced RNA editing, particularly changes in editing patterns is substantially changed in the prefrontal cortex of patients with depression and schizophrenia suggesting its role in neuropsychological disorders (Eran et al., 2013; Mombereau et al., 2010). *C. elegans* strains with homozygous deletion of both ADAR genes, *adr-1*, *adr-2*, have chemotactic malfunction (Tonkin et al., 2002).
There are two major effects of A-I editing:

1) A-to-I editing changes the information content of RNA molecules, since inosine (I) is preferentially binding to C, so the splicing and translating machinery are interpreting it like G;

2) A-I editing changes tridimensional structure of double stranded RNA with introduction or removal of single stranded bulges, that result from imperfect base pairing, and the tridimensional structure alone of double stranded RNA determines its interaction with RNA binding proteins.

Editing efficacy of substrates (number of converted A to I) is varying depending on the type of substrate, development phase and physiological condition, that allows existence of diverse products, and a turnover of products as a response to changed environmental conditions.

Unlike ADARs, ADATs lacks the dsRBD therefore, their recognition of substrate is mainly dependent on the tertiary structure of tRNA. ADATs similarly to ADARs, possess the deaminase domain and it is suggested that ADARs evolved from ADATs with fusion of dsRBDs (Keegan et al., 2004).
ADAT1 acts as homodimer introducing inosine on position 37, located after the anticodon in eukaryotic tRNA(Ala). After this reaction tRNA methyltransferase 5 (Trm5) introduces methylation at N1 position of inosine resulting in final m1I37 (Grosjean et al., 1995a; Grosjean et al., 1995b). ADAT1 is localized mainly in the nucleus where studies suggest it may interact with a dsRBD containing protein Disconnected Interacting Protein 1 (DIP1) and acts at pre-tRNA level (Catanese and Matthews, 2011). ADAT2 and ADAT3 form a heterodimer to catalyze the deamination of adenosine on position 34, located at first position of the anticodon (wobble-position) on 8 cytosolic tRNAs (Torres et al., 2015). ADAT2 is the catalytic subunit while ADAT3 probably is responsible for substrate recognition (Gerber and Keller, 1999). ADAT2 and ADAT3 localize mainly in the nucleus but their presence in the cytosol is not excluded, as it was shown

that ADAT3 located in the cytoplasm can translocate to the nucleus in presence of ADAT2 (Torres et al., 2015). In Prokaryotes I34 is introduced by Tad1 homodimer, only on tRNA(Arg). The enzyme catalyzing deamination of adenosine on position 57 in Archaea is not known (Grosjean et al., 1995a; Yamaizumi et al., 1982).

Deficiency in inosine synthesis on tRNAs has not been extensively investigated. Yeast knockouts for Tad2 (ADAT2) or Tad3 (ADAT3) are lethal. Tad1 (ADAT1) yeast knockout strains are viable and mutants with disrupted *Tad1* show no particular growth impairment, suggesting m1I37 and I37 modification are not essential in yeast (Gerber et al., 1998).

The ADAT1 transcript was found to localize mainly to the central nervous system during *D. melanogaster* development, suggesting a possible role for ADAT1 and m1I37 modification during brain development (Keegan et al., 2000). In humans, it has been reported that both I34 and m1I37 of tRNA(Ala), are targets of autoantibodies generated against the anticodon stem loop of tRNA(Ala) in serum of patients suffering from myositis, a chronic inflammatory muscle disorder (Becker et al., 1999).

4.1.1.3.4. Erasers

Inosine specific erasers have not been reported yet.

4.1.1.3.5. Roles of readers and A-to-I editing by ADAR

Most frequently editing locations are long, partially complementary RNAs formed from inverted non-coding repeats, such as Alu and **L**ong **in**terspersed **e**lement (LINE) located in mRNA UTRs and introns. Globally editing of non-coding RNA could control gene expression that contains these repeated sequences affecting: alternative splicing, nuclear retention, transcript degradation, silencing of heterochromatin or suppression of small regulatory RNA (piwi-interacting RNA). RNA editing regulates also processing and expression of mature miRNA, while ADAR1L traps siRNA, decreasing RNA interference (RNAi) efficacy.

- Protein sequence recoding – Regulation of protein function

Before splicing of eukaryotic transcript, the region between intron and exon in precursor messenger RNA often forms stem-loop structures. These structures can be edited with ADAR, and the formed Is are read as Gs by ribosomes, since they are base-pairing with Cs. Thus, any editing in the protein coding region can lead to an amino acid change and this type of editing is known as

recoding-type editing (Hood and Emeson, 2012). Around 30 genes for proteins undergo editing. Well-investigated examples of mRNA editing are receptor for glutamate (AMPA, GluR-B) and receptor for serotonin type 2C (5-HT2CR) in mammals that contain several specific editing positions resulting in production of protein isoforms of different functionality (Burns et al., 1997; Higuchi et al., 1993).

L-glutamate is the predominant excitatory neurotransmitter in the nervous system of vertebrates and its receptors play roles in neuronal plasticity and complex functions like memory and studying. AMPA receptor for glutamate that is also an ion channel for calcium (Ca), takes part in the rapid excitatory transmission in central nervous system (CNS) neurons and it contains 4 subunits, referred as GluR-A to D. mRNA for GluR-B subunit (Gria2) undergoes ADAR2-A-to-I editing, so the codon for glutamine (Q) in exon 11 changes into codon for arginine (R). This editing changes significantly AMPA receptor (GluR-B) Ca ions permeability: arginine presence blocks Ca conductance through channel, while the existence of genomic encoded Q allows Ca influx in cells (Higuchi et al., 1993).

Serotonin has an important role in physiological process and behavior, like circadian rhythm, control of emotions and food related behavior. mRNA of 5-HT2cR is edited in 5 specific places in exon 5 coding a long intramolecular loop in the domain for G protein coupling. Different editing combinations of these 5 sites lead to a change of three codons into possible six new ultimately resulting in expression of 24 different receptor isoforms of different functionality (Burns et al., 1997).

Three possible patterns of this functional modulation via editing are known.

1) Editing of all 5 sites (A, B, C, D and E) changes genome encoded amino acids Ile, Asp and Ile on positions 156, 158 and 160, each of them leading to a change in coupling efficacy with G protein. Completely edited isoform (5-HT2CR-VGV) has reduced potentiation with serotonin, coupling with G protein and agonist binding in comparison to non-edited isoform (5-HT2CR-INI).

2) Editing pattern controls amount of 5-HT2CR mRNA that leads to expression of full-length protein through modulation of selection of alternative splicing sites. Between three alternative 5′- splicing sites (GU1, GU2 and GU3), use of GU2 site gives full length functional 5-HT2CR protein. Non-edited pre-mRNA uses generally GU1 splice site that in

case of translation gives rise to a nonfunctional protein. However, most of edited pre-mRNA are spliced from GU2 site. Therefore, if editing is inefficient, increased splicing in GU1 site works as a control mechanism for decreasing synthesis of 5-HT2CR-INI that limits serotonin response.

3) RNA editing controls the ultimate physiological output of constitutively active receptors affecting expression of surface 5-HT2CR on cell surface. 5-HT2CR-VGV, that possess the lowest level of constitutive activity, is fully expressed on cell surface in basal conditions and it internalizes fast upon agonist presence. On the contrary, 5-HT2CR-INI is constitutively internalized and is accumulated in endosomes.

Some sites are specifically edited with ADAR1, some with ADAR2, while others are not showing specificity for one or the other enzyme. For instance, A, B sites 5-HT2CR are selectively edited with ADAR1 while site D with ADAR2 (Burns et al., 1997; Fitzgerald et al., 1999; Flomen et al., 2004; Niswender et al., 1998).

- Changes in splicing sites

Intron regions can also undergo editing, and that can affect splicing, since splicing machinery can interpret I as a G. For this reason, A-I editing has the potential of creating a 5′-splice site, creating or eliminating 3′-splice site and removing branching site. For instance, GU in 5′-splice site can be created due to editing AU-> IU=GU, likewise AG in 3′-splice site (AA->AI=AG).

This is clearly evidenced in ADAR2 self-regulation that edits its own double-stranded intronic RNA creating an alternative 3′-splice site. Translation of alternatively spliced ADAR2 mRNA produces an incomplete protein lacking RNA binding domain and deaminase domain thus autoediting leads to ADAR2 repressed expression (Rueter et al., 1999).

- Effects in noncoding sequences

There are some evidences that editing of Alu repeats, often localized in 3′ UTRs of mRNA, leads to preferential retention in the nucleus through binding of paraspeckle regulatory protein 54-kDa nuclear RNA-binding protein (p54nrb), as it was shown for the mRNA of cationic amino acid transporter 2 (Cat2). Once edited Alu elements in 3′ UTR form a binding site for p54nrb that keeps the mRNA in the nuclear speckles. In stress conditions, Cat2 mRNA is posttrancriptionally cut and *de novo* polyadenilated on an alternative site resulting in transport of Cat2 mRNA in the cytosol and translation into protein (Prasanth et al., 2005).

Another mechanism for regulating gene expression is the presence of ribonuclease specific for RNA containing inosine that cuts both strands of RNA with high numbers of I-U mismatches (Scadden and Smith, 2001b). Tudor staphylococcal nuclease (Tudor-SN) was until recently a candidate for targeting of pre-mRNA edited in their inverted Alu and LINE repeats. However, Endonuclease V (EndoV) was recently identified as this ribonuclease and Tudor-SN acts as a cofactor. Thus, this possible Tudor-SN-EndoV degradation would control the level of expression of genes containing these sequences (Morita et al., 2013; Scadden, 2005).

- Crosstalk between editing and RNAi

RNAi and A-to-I editing are two pathways known to interact. In essence of this interaction is the competition of editing A-I and RNAi enzymes for double-stranded RNA substrates. Small regulatory RNAs are playing a role in many diverse cellular processes, where most of them are double-stranded during their biogenesis, suggesting that ADAR's editing could have a regulatory role in many processes.

Two recognized models of interaction between RNA editing and RNAi involving endo-siRNA are evidenced:

1) Editing of A-to-I double-stranded endo-siRNA precursor making it more resistant to Dicer activity;

2) Supression of endo-siRNA with ADAR1L, where ADAR1L binds tightly to endo-siRNA and decreases the effective endo-siRNA concentration;

both models result in decreased RNAi efficacy.

RNAi can be induced with long dsRNA molecules as substrates for Dicer. Post Dicer processing produces siRNA that can direct endonucleolytic degradation of the original transcript or any other RNA with identical sequence. Long dsRNAs are also a substrate for ADAR, where once I-U mismatches are formed they affect the structure of double stranded RNA making it more resistant to Dicer activity and producing less endo-siRNA. It is believed that Dicer can differentiate between perfectly based paired RNA and edited RNA containing I-U pairs, as *in vitro* studies suggest extensive editing suppresses cutting of double stranded RNA with Dicer thus reducing RNA interference (Scadden and Smith, 2001a).

C. elegans mutants carrying homozygous deletions of *adr-1* and *adr-2* genes show defects in chemotaxis (problems in finding or avoiding certain substances) (Tonkin et al., 2002). Reversion

of this phenotype is possible in those worms having a defect in RNAi machinery. Altered chemotactic phenotype in *C. elegans* is resulting from hyperactivity of RNAi pathway, which in normal conditions is suppressed by ADAR. Expression of genes controlling chemotaxis depends of the balance between A-I editing and RNAi acting on dsRNA formed in the transcripts of these genes (Tonkin and Bass, 2003).

Besides the competition with Dicer for dsRNA substrates, ADAR1L binds tightly to processed ds endo-siRNA, decreasing its concentration in the cytoplasm and reducing RNAi efficacy. This is confirmed with significantly more efficient gene silencing with siRNA in absence of ADAR1 (Heale et al., 2009; Warf et al., 2012; Yang et al., 2005). Expression of ADAR1 in mice is induced when unspecific siRNA are injected in high amounts, suggesting ADAR1 is a part of a cellular mechanism as a response to siRNA (Hong et al., 2005).

- Effects on miRNA pathway

A-to-I editing has been found in many endogenous primary RNA (pri-miRNA) and there are *in vitro* evidences of precursor miRNA editing (pre-miRNA), suggesting editing might modulate miRNA processing and thereby regulate cell miRNA levels.

Editing of pri-miRNA can have major impact on biogenesis and expression of miRNA considering that short and imperfect double stranded regions of pri- and pre-mi RNA allow ADAR enzymes to take part in the miRNA biogenesis (Yang et al., 2006). ADAR editing can affect miRNA processing inhibiting endonucleolytic processing by Drosha or Dicer, resulting in decreased amounts of mature miRNA (Kawahara et al., 2007). Highly-edited pri-miRNA are further degraded with Tudor-SN-EndoV complexes (Morita et al., 2013). In case the miRNA processing is not compromised, a mature miRNA is expressed, containing A-to-I substitutions representing an edited mature miRNA. These miRNAs can silence a set of genes that is different from the set of genes their non-edited copies would target, especially if the editing was in the “seed” region of miRNA, crucial for recognition of target mRNA (Ekdahl et al., 2012; Kawahara et al., 2007).

In most of the cases one guide strand of ds miRNA is selected in the RNA-induced silencing complex (RISC) while the passenger strand is degraded. This step in miRNA biogenesis can also be modulated with A-to-I editing. Selection of the guide strand is based on thermodynamical stability of miRNA 5′-end and editing can change local stability of ds miRNA. Editing induced structural changes may affect the guide strand selection majorly affecting the choice of target mRNA (Kume et al., 2014).

3′ UTRs in mRNA are also subjected to editing. Changing A into I in 3′ UTR makes a new target site for miRNA, thus 3′ UTR editing can increase or repress silencing of specific mRNA. Similarly, editing can destabilize secondary structures in 3′ UTR allowing RISC complex to interact with previously unavailable sites (Zhang et al., 2016b).

4.1.1.3.6. Role of ADAT in RNA function and fate

Codon-anticodon recognition is essential for amino acid incorporation into proteins. Wobble nucleoside A34 can pair with U while I34 is capable of pairing with A, U and C. This ability of inosine to pair with more nucleosides suggest I34 increases number of codons recognized by a tRNA. Since I is recognized as G, codons having C at their end are possibly affected by the presence or absence of the I34 modification. Presence of A34 tRNAs and G34 tRNAs were shown to be exclusive in the same genome suggesting I34 in tRNA is important in reading C-ended codons (Grosjean et al., 2010). Most expressed genes show to have a preference for specific codons resulting in increased translation efficiency (Gustafsson et al., 2004). Since anticodon modifications may affect codon recognition by tRNA, changes in ADATs activity and modification status of tRNAs could have an impact on translation of specific genes.

4.1.1.4. *2′-O-methylation of ribose*

4.1.1.4.1. Discovery of 2′-O-methylation

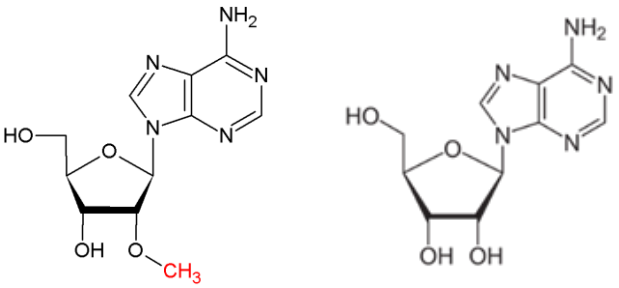

Figure 7. Structure of Am (left) and adenosine (right). Adapted from URL: http://modomics.genesilico.pl/modifications/Am/

Methylation of the 2′-hydroxyl group of the ribose (Figure 7) is a predominant internal modification of rRNA and spliceosomal RNA. Mammalian rRNA carries 105-107 2′-O-methylated residues while it has been reported that the spliceosomal snRNAs (U1-U6 snRNAs) bear a total of 30 2′-O-methylated residues (Maden, 1990; Reddy and Busch, 1988). SnoRNA U3 has also abundant 2'-O-methylations (Reddy and Busch, 1988). 2'-*O*-methylation is identified in all tRNAs (Grosjean et al., 1995b). Additionally, small noncoding RNA, including piwi-interacting RNAs (piRNAs), endogenous small interfering RNAs (endo-siRNAs) and plant miRNAs, are 2′-O-methylated at their 3′-ends by methyltransferases (Horwich et al., 2007; Pelisson et al., 2007; Yu et al., 2005). This 3′-

methylation in the small RNAs prevents terminal uridylation (Li et al., 2005) and degradation by exonucleases (Ramachandran and Chen, 2008) possibly playing a role in specific RNA interference (RNAi) pathways. All eukaryotic mRNAs contain a 5′ cap structure that carries a methyl code. This methyl code contains m^7G - cap 0, and it can additionally possess one or both of the first nucleotides methylated on the 2´-hydroxyl group to form 'cap-1' or to form 'cap-2', respectively (Langberg and Moss, 1981).

4.1.1.4.2. Detection of 2′-O-methylation

2′-O-methylation as a sugar ring modification changes the biochemical properties of the modified nucleotide compared to the unmodified nucleotide. This methylation favors 3′- endo axial conformation of the sugar and changes the hydration sphere around the 2′-O thus affecting RNA structure (Auffinger and Westhof, 1997; Auffinger and Westhof, 1998). Phosphodiester bonds adjacent to 2′-O-methyl groups are resistant to alkaline hydrolysis (Maden, 2001). Similarly, RNaseH non-specific cleavage of RNA-DNA hybrids is prevented in presence of 2′-O-methylation on a RNA residue (Yu et al., 1997). These specific features have been exploited for mapping 2′-O -methylation extensively in rRNA and tRNA.

Diverse biochemical approaches have been developed to detect and quantify 2′-O-methylation. Initial studies relied on liquid chromatography coupled with mass spectrometry (LC/MS) (Douthwaite and Kirpekar, 2007) and two-dimensional thin-layer chromatography (2D-TLC) approaches (Grosjean et al., 2004). However, these procedures were found quite demanding in terms of preparation and purification of starting material. To avoid tedious RNA purification, primer extension by reverse transcriptase was favored as a faster approach. In this methodology the RNA of interest is specifically selected through the annealing reaction with specific oligonucleotide DNA primers. Three main variations of the standardized reverse transcription (RT) reaction have been developed: (1) conditions of low dNTP concentration where reverse transcriptase stalls on 2′-O-methyl groups (Maden et al., 1995), (2) the use of partial alkaline hydrolysis followed by RT (Kiss-Laszlo et al., 1996) and (3) **R**everse **T**ranscription at **L**ow dNTP concentrations followed by **P**CR (RTL-P) (Dong et al., 2012). RTL-P represents the most advanced modification of RT to detect 2′-O-methylation, able to detect both previously identified and novel 2′-O-methylated sites in human and yeast rRNAs, as well as mouse piwi-interacting RNAs (piRNAs), without requiring substantial amount of initial material (Dong et al., 2012). Site-

specific methodologies based on the resistance of 2′-O-methylated sites to cleavage by RNase H have also been developed (Lapham et al., 1997; Yu et al., 1997). In this methodology a specific DNA-RNA chimera oligonucleotide is constructed to base-pair with the target RNA, where the "chimera" consists of a DNA oligonucleotide, hybridizing to the target RNA, and flanking 2′-O-methyl ribonucleotides. RNase H will cleave target RNA at the phoshodiester bond 3′ to the ribonucleotide that is base-paired with the 5′- most deoxynucleotide of the chimera. RNase H will not cleave a bond adjacent to a 2′-O-methyl in target RNA. Therefore, target RNA containing 2′-O-methylation will result in a single large product whereas in absence of 2′-O-methylation two products will be detected on gel electrophoresis. RNAse H based approach is quantitative approach and it requires a radiolabeling step (Lapham et al., 1997; Yu et al., 1997). More recent approaches are high-throughput methods based on sequencing of different depth. Ribometh-seq detection is based on random RNA fragmentation under alkaline conditions and library preparation followed by next-generation sequencing (NGS). By randomly hydrolyzing RNA and performing NGS at very high depth, uniform coverage of 3′-end positions is expected across regions of interest except at positions of 2′-O-methylation. This "negative" selection of RNA 2′-O-methylation can then be converted into a peak diagram and a methylation score expressing the fraction of molecules methylated at a specific site (Krogh et al., 2017).

Ribose oxidation sequencing (RibOxi-seq) employs Benzonase nuclease for RNA degradation instead of alkaline conditions. Fragmented RNAs containing 3′-ends that are either unmethylated or 2′-O-methylated undergo an oxidation step where 2′ and 3′-hydroxyls of non-2′-O-methylated riboses are converted into dialdehydes using sodium periodate (NaIO4), thus preventing them from being ligated to linkers in subsequent sequencing library construction. Only samples being "positive" for 2′-O-methylation will be included in the sequencing library. After sequencing, the reads are aligned to a reference genome and only positions of the 3′-ends of aligned fragments are evaluated. After normalizing the data is analyzed using DESeq2 for single-base resolution methylation site determination (Zhu et al., 2017).

4.1.1.4.3. Writers

Introducing 2′-O-methylation on ribose in rRNA, mRNA and tRNA is mediated by complexes of guide RNA and proteins named Small nucleolar ribonucleoproteins (snoRNP) complexes (Figure 8) or solely methyltransferases.

- SnoRNP

SnoRNP complexes consists of proteins: Nop1p (the catalytic component, also known as fibrillarin in human), Nop56p, Nop58p and Snu13p subunits (Lafontaine and Tollervey, 1999; Schimmang et al., 1989; Wu et al., 1998), guided by Box C/D small snoRNAs to the appropriate base (Balakin et al., 1996; Kiss-Laszlo et al., 1996). Fibrillarin (Nop1p) shares a conserved domain with known SAM-dependent methyltransferases, suggesting that fibrillarin is the methyltransferase in box C/D snoRNPs (Wang et al., 2000a).

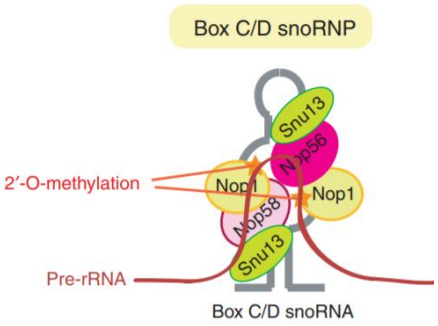

Figure 8. SnoRNP complex introducing 2′-O-methylation in pre-rRNA. Adapted from (Kakihara and Saeki, 2014).

Box C/D snoRNA were identified in studies where substantial complementarity has been detected with rRNA, suggesting their role in ribosome biogenesis (Bachellerie et al., 1995). Additionally, Bachellerie (et al.) found that methylation sites were closely located to complementary regions between rRNA and snoRNA suggesting that box C/D snoRNAs function as guides for 2'-O-methylation of rRNA. Tollervey (et al.) reported that certain mutations in the yeast Nop1 homolog resulted in reduced methylation of pre-rRNA (Tollervey et al., 1993). Soon after rRNA 2'-O-methylation was localized 5 nucleotides upstream from box D (or D') in the residue base-paired to the nucleotide in snoRNA (Cavaille et al., 1996). Deletion of a particular box C/D snoRNA resulted in loss of 2'-O-methylation at the target site in rRNA in yeast. However, site-specific methylation could be restored upon reintroduction of the box C/D snoRNA into the deletion strain (Kiss-Laszlo et al., 1996), and modification of novel target sites could be directed by introduction of snoRNAs with appropriate guide sequences (Cavaille et al., 1996).

The 2′-O-methylation guide snoRNAs possess the conserved C (consensus RUGAUGA) and D (CUGA) boxes, that can be folded together with an adjacent 5′, 3′-terminal stem, and incomplete copies of the C and D boxes called C′ and D′ boxes in the internal regions of box C/D snoRNAs. Positioning of D or D’ box exactly 5 nt from the future 2′-O-methylated ribosomal nucleotide is supported through formation of double helices between rRNA sequences and the recognition sequence (10–21 nt long) that precedes D and/or D′ boxes (Cavaille et al., 1996; Kiss-Laszlo et al., 1996). The C/D boxes are required for snoRNA stability and localizing snoRNAs to the nucleolus, which was confirmed with findings indicating snoRNA synthesis and localization are

coupled (Samarsky et al., 1998). In addition to snoRNAs, cells contain small Cajal body-specific RNAs (scaRNPs) that can possess a C/D box but they contain additional elements that retain them in Cajal bodies (CBs). Many of them target modifications of spliceosomal snRNA (Jády et al., 2003).

- Methyltransferases

Human cap1 2′-O-ribose methyltransferase, hMTr1, catalyzes the methylation of the 2′-O-ribose of the first transcribed nucleoside of a capped RNA. Its methyltransferase activity was shown not to be dependent on guanosine N^7-methylation of the cap structures (Belanger et al., 2010). The product encoded by the hMTr1 gene has been previously described (called ISG95), however its role in cap1 formation was underinvestigated. In that study authors noted ISG95 associates with the C-terminal domain of RNA polymerase II, suggesting that cap1 methylation occurs co-transcriptionally (Haline-Vaz et al., 2008).

Cap2 MTase in human is hMTr2 encoded by *HMTR2* gene, similarly to hMRT1 and its methylation activity shows no preference between m^7GpppN and GpppN structures. However, hMTr2 is less efficient for RNA without cap1. Cellular localization of hMTr1 is nuclear, and hMTr2 is present in the cytoplasm and the nucleus (Werner et al., 2011).

2′-O-methylation of adenosine at residue 4, identified only in tRNA(His), is catalyzed by protein Trm13 protein in *S. cerevisiae* (Wilkinson et al., 2007).

4.1.1.4.4. Erasers

Enzymes capable of “erasing” 2′-O-methylation have not been identified.

4.1.1.4.5. Readers

Proteins associating with cap1 and cap2 including the translation machinery are known to recognize 2′-O-methylation in mRNA. However, proteins recognizing 2′-O-methylation in other positions than 5′ cap have not been identified.

4.1.1.4.6. Role of 2′-O-methylation in RNA function and fate

Distribution of 2′-O-methylation within the ribosome is restricted to functionally relevant regions of 40S and 60S. Most of the structural and functional studies to investigate the role of ribosomal 2′-O-methylation were conducted in *E. coli* and *S. cerevisiae*. In *S. cerevisiae* the positions of 2′-O-methylation confine to the decoding and tRNA binding sites (the A-, P- and E-sites), the

peptidyltransferase center (PTC site) and the intersubunit bridge. This defined spatial distribution has been conserved throughout evolution although the abundance and locations of these nucleotides within functional regions vary phylogenetically (Decatur and Fournier, 2002; Rozenski et al., 1999). Their high concentration in functionally important regions of the ribosome reflects either direct or indirect contribution to ribosome activity. Direct contribution of 2′-O-methylation is probably due to maintenance of secondary and tertiary structures owing to stabilization of helices by increasing base-stacking (Auffinger and Westhof, 1997; Auffinger and Westhof, 1998; Decatur and Fournier, 2002). Through structural stabilization of ribosomes most likely it indirectly mediates translation (Decatur and Fournier, 2002). Early studies using catalytically inactive Nop1 mutants demonstrated the importance of 2′-O-methylation for cell growth and ribosome production in yeast (Tollervey et al., 1993). Additionally, it has been proven in yeast that Nop1 can be functionally complemented with expression of human or *X. laevus* fibrillarin indicating that its function has been conserved from yeast to vertebrates (Jansen et al., 1991). Deletion of each box C/D snoRNA individually in cells revealed phenotypes with subtle defects in translation and growth (Esguerra et al., 2008).

In addition, discoveries of new snoRNAs, also known as ‘orphan’, are predicted to target other RNA species than ribosomal RNAs or small non-coding RNA (Jorjani et al., 2016). In particular, a brain-specific snoRNA C/D box (SNORD) 115, named MBII-52 may guide 2′-O-methylation of the serotonin receptor 5-HT2C pre-mRNA, thereby regulating gene expression. Importantly, the nucleotide that is predicted to be modified by MBII-52 C/D RNA is precisely the ADAR2 edited C-site within 5-HT2C pre-mRNA. A 2′-O-methylation of adenosine was shown to specifically decrease the deamination rate of the modified adenosine *in vitro* (Yi-Brunozzi et al., 1999). Therefore, MBII-52 negatively regulates ADAR2-mediated nucleolar editing of an RNA substrate in a sequence-specific manner (Vitali et al., 2005).

Presence of cap1 2′-O-methylated mRNA represents a signature of “self” preventing recognition through retinoic acid inducible gene I (RIG-I) receptor (Devarkar et al., 2016; Schuberth-Wagner et al., 2015). Cap2 methylation may lead to increased translation of mRNA by promoting its stability (Werner et al., 2011). Many viruses possess mRNA 5′-structures or encode functions associated with the formation of a 5′ cap that are homologous to those found in eukaryotic cells e.g.: RNA 5′-triphosphatase, RNA guanylyltransferase, RNA guanine-N7-methyltransferase (N7-methyltransferase), and 2′-O-methyltransferase (Furuichi and Shatkin, 2000). It has been reported

that human and mouse coronavirus mutants lacking 2′-O-methyltransferase activity induced higher expression of type I interferon compared to wild-type strains and that this induction was dependent on the cytoplasmic RNA sensor MDA5. This defines 2′-O-ribose methylation in mRNA as a molecular marking that helps our immune system to distinguish between self and non-self mRNA (Zust et al., 2011).

U2 snRNA cap1 and cap2 methylations are required for spliceosome E complex formation and ultimately for efficient pre-mRNA splicing (Donmez et al., 2004).

4.1.2. Cytosine related modifications

4.1.2.1. ***5-methylcytosine (m5C)***

4.1.2.2. Discovery and detection of m5C

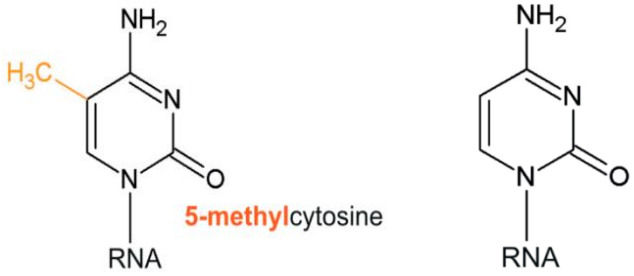

Figure 9. Structure of m5C (left) and cytosine (right). Adapted from (Van Haute et al., 2017)

Cytosine base methylation 5-methylcytosine (m5C) (Figure 9) has been identified in rRNA, tRNA and recently in mRNAs, particularly enriched in the untranslated regions and near miRNA binding regions (Squires et al., 2012). This has been facilitated in large part by the ease of its detection in DNA using bisulfite sequencing, which involves chemical conversion of cytosine (but not m5C) to uracil (Lister et al., 2009). Bisulfite sequencing adapted for RNA bisulfite conversion in combination with next generation sequencing further identified m5C in both coding and non-coding RNAs in addition to tRNAs and rRNAs (Squires et al., 2012).

4.1.2.3. Writers

Enzyme responsible for introduction of m5C are the DNA methyltransferase homolog (Dnmt2) and members of the NOP2/Sun (NSun) RNA methyltransferase family.

While Dnmt2-mediated methylation of cytosine 38 in the anticodon loop of tRNA(Asp) is highly conserved, plants, insects and mice lacking Dnmt2 protein show no morphological defects (Goll et al., 2006). However, in zebrafish morpholino-mediated loss of Dnmt2 was shown to impact on late differentiation resulting in half-sized morphants with liver, brain and retina defects (Rai et al., 2007). In *D. melanogaster*, overexpression of Dnmt2 was evidenced to increase lifespan and resistance to stress (Lin et al., 2005) while loss-of-function mutants had reduced viability in

thermal and oxidative stress conditions. Moreover, in these mutants stress-induced cleavage of tRNA was reduced upon ectopic expression of Dnmt2 (Schaefer et al., 2010).

NSun2 and NSun4 are members of the SAM-dependent methyltransferase superfamily and both are able to introduce 5-methylcytidine in RNA in mammals (Frye and Watt, 2006; Metodiev et al., 2014). Deletion of Nsun2 in mice, similarly to Dnmt2 loss in zebrafish causes defects in late differentiation affecting growth and specific tissues including skin and testis (Blanco et al., 2011; Hussain et al., 2013). Lack of tRNA methylation in absence of Dnmt2 and Nsun2 impairs differentiation, particularly of brain, liver and adipose tissue (Tuorto et al., 2012). Human *Nsun2* gene mutations have been identified to cause autosomal-recessive intellectual disability and symptoms similar to the phenotype observed in *D. melanogaster* lacking Nsun2 in Dubowitz-like syndrome. These symptoms include growth and mental retardation and cutaneous abnormalities (Abbasi-Moheb et al., 2012; Martinez et al., 2012). The role of RNA methylation in these complex diseases is currently unknown.

Nsun4 is responsible for cytosine (C911) methylation of 12S rRNA in mitochondria. Unlike, Nsun2 deletion, Nsun4 germline knockout was shown to be lethal and with embryos at E8.5 showing growth retardation and lacking anatomical structures. Nsun4 heart-specific deletion affects not only methylation of 12S rRNA but also interaction with mitochondrial transcription termination factor-4 (MTERF4) important for mitoribosome assembly and impaired respiratory chain biogenesis resulting in mitochondrial dysfunction and cardiomyopathy (Metodiev et al., 2014).

Protoncogene c-Myc was shown to transcriptionally target Nsun2 in mammalian epidermis. Upregulated expression of Nsun2 is possibly implicated in wide variety of cancers. Knockdown of Nsun2 in human squamous-cell-carcinoma xenografts were shown to decrease their growth (Frye and Watt, 2006). Nsun2 is a substrate of Aurora kinase B and independently of its methyltransferase activity, has a potential in stabilizing mitotic spindle and promotion of cell division in cancer cell lines (Hussain et al., 2009; Sakita-Suto et al., 2007).

4.1.2.4. Erasers and readers

m5C-demethylases and m5C specific readers have not been reported yet.

4.1.2.5. Role of m5C in RNA function and fate

Functions of RNA m5C methyltransferases are still poorly understood. The link to human diseases may be explained by a direct role of m5C in rRNA and tRNA to regulate global protein translation through structural stabilization. However, the potential roles of m5C in mRNA remain still to be investigated.

4.1.3. Uridine related modification

4.1.3.1. *Pseudouridine*

4.1.3.1.1. Discovery of Pseudouridine

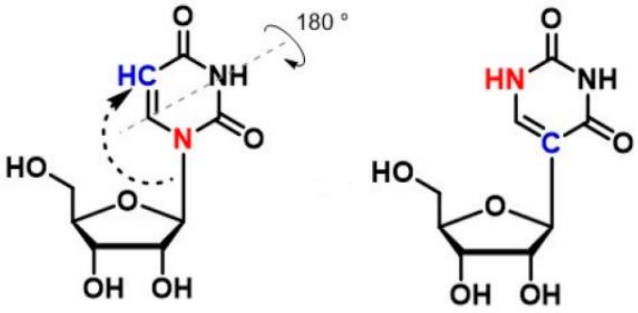

Figure 10. Structure of uridine (left) isomerization into Ψ (right). Adapted from (Penzo et al., 2017).

Six decades ago the fifth nucleoside was identified in yeast (Davis and Allen, 1957). Soon after this fifth base was named Pseudouridine (Ψ) (Cohn, 1959), and currently is one of the abundant nucleoside modification present in all three life domains (Machnicka et al., 2013). After its initial detection in rRNA and tRNA, Ψ was found in mRNA, lncRNA, snRNA, like U2 snRNA, and snoRNA (Kim et al., 2010; Schwartz et al., 2014a). In yeast, Ψ is present in many positions in cytoplasmic and mitochondrial tRNAs, around 50 across the four rRNAs (25S, 18S, 5.8S, and 5S), and in six positions in U1, U2, and U5 snRNA (Charette and Gray, 2000; Ofengand, 2002; Yu et al., 2011). Studies in HEK293 and human fibroblast confirmed these conserved Ψ profiles in human, but the number of Ψ increases with around 100 Ψ found per one human ribosomes (Schwartz et al., 2014a).

4.1.3.1.2. Detection of Ψ

Pseudouridylation results from enzymatic isomerization (an internal transglycosylation) of a uridine in an RNA molecule (Figure 10). This isomerization results in the change of C-N glycosidic bond to a more inert C-C bond (Cohn, 1959) and presence of two NH imino protons available to serve as hydrogen bond (Davis, 1995). Such structural changes enhance base stacking and rigidity of the phosphodiester backbone resulting in more stable pairing with any of the 4 major bases than their U equivalents (Arnez and Steitz, 1994; Davis, 1995; Kierzek et al., 2014). Nuclear magnetic

resonance (NMR) studies of tRNA indicated an involvement of Ψ's N1-H proton in a stable hydrogen bond, even within putative single-stranded regions (Roy et al., 1984).

Uridine and Ψ share a similar UV spectrum and identical molecular mass (Yu and Allen, 1959), and during RT its pairing with adenosine is possible due to Watson–Crick pairing with an unmodified template leaving no traces in cDNA. Thus, being mass- and RT-silent, detection of Ψ has been hampered for decades. Main methodologies in mapping Ψ were a combination of RNAse digestion, radiolabeling, and chromatography-based methods (Gupta et al., 1979; Holley et al., 1965; Tanaka et al., 1980). Owing to the possibility of chemically modify Ψ with N-cyclohexyl-N′-β-(4-methyl-morpholinium) ethylcarbodiimidep-tosylate (CMC), a novel approach has been developed and it is still the basis of current most sensitive detection methods (Bakin and Ofengand, 1993). CMC under physiological conditions acylates guanosine and uracile at N1 and N3 positions, respectively. The acylation reaction upon CMC treatment in Ψ occurs on both N1 and N3 positions. With alkaline conditions (pH=10.4), CMC adducts undergo hydrolysis except on N3 position of Ψ leaving CMC irreversibly bound. This blocks RT one base downstream of Ψ and helps its detection. However, not all Pseurdouridines are equally 'marked' due to incomplete acylation with CMC. Similarly, the hydrolysis from U and G residues can be partial and result in false positives. After CMC treatment of RNA, RT-stalls on CMC-acylated G, U and Ψ residues. Since alkaline hydrolysis removes CMC from G and U residues, Ψ's CMC residues resists hydrolysis under these conditions and therefore can be detected as RT-stopping signals. Recently, in four sequencing approaches: Pseudo-seq, Ψ-seq, Pseudouridine site identification sequencing (PSI-seq), and N3-CMC-enriched pseudouridine sequencing (CeU-seq), for Ψ mapping in yeast and human transcriptomes a combination of this chemical treatment and RT has been exploited. Sequencing approaches applying RT-CMC differ in enrichment of initial Ψ-containing transcripts, library preparation and bioinformatical analysis (Carlile et al., 2015; Li et al., 2015; Lovejoy et al., 2014; Schwartz et al., 2014a).

In principle polyA-RNA fractions undergo CMC treatment followed by alkaline hydrolysis. Then, an adaptor is ligated to the 3′ end of RNAs and transcripts are reverse transcribed. Ψ-CMC-induced RT arrest results in truncated cDNA products. Depending on the method, 3′ adaptors are ligated to the resulting cDNAs or RT products that were circularized for PCR amplification and deep sequencing. Control libraries are constructed in parallel from samples without CMC treatment. In Ψ-seq and PSI-seq polyA-RNAs is fragmented to a uniform size range selected prior to CMC

treatment and RT. Upon alkaline hydrolysis CMC will be removed from uridines and guanosine and remaining on Ψ in treated samples. Truncated cDNA from treated samples will contain a reproducibly enriched set of fragments due to CMC marking and following RT stopping. CeU-seq (N3-CMC-enriched Ψ sequencing) enriches the fraction of CMC-labeled transcripts by applying CMC-azide derivative which allows biotin conjugation with click chemistry following derivatization and subsequent hydrolysis. Ψ-CMC-biotin containing transcripts are then pulled down with streptavidin beads, increasing the method's sensitivity with a benefit of approximately 15–20-fold enrichment of pseudouridylated RNAs. PSI-seq unlike other sequencing approaches relies solely on bioinformatical analysis without any enrichment steps (Carlile et al., 2015; Li et al., 2015; Lovejoy et al., 2014; Schwartz et al., 2014a).

4.1.3.1.3. Writers

Introducing Ψ in eukaryotic RNA can be mediated through RNA dependent pseudouridine synthases (PUSs) or RNA-independent PUSs.

RNA dependent PUSs are SnoRNP complexes consisting of H/ACA snoRNA that guide catalytically active proteins to target site via base-pairing to target RNA (Figure 11).

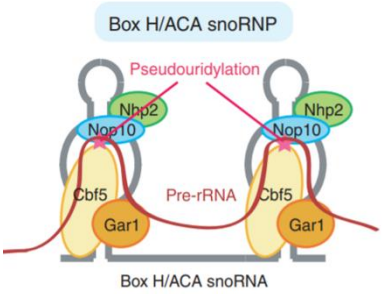

Figure 11. SnoRNP complex involved pseudouridylation in pre-rRNA. Adapted from (Kakihara and Saeki, 2014).

SnoRNA H/ACA possess hairpin-hinge-hairpin-tail structure with the hinge region containing the conserved sequence block ANANNA (box H) and the ACA motif found 3 nucleotides away from the 3′-end (Ni et al., 1997). Specific base-pairing with target RNA is mediated through two short guide sequences in a loop part of the duplex structures with 14-15 nt distance from H or ACA motifs. Upon transient base-pairing of target RNA, uridine is placed in a pocket between the flanking paired regions where it undergoes isomerization. The canonical target of a majority of snoRNAs is rRNA, but snRNAs are also modified through a distinct population of snoRNAs known as small Cajal body-specific RNAs (scaRNAs) (Wu et al., 2011b).

SnoRNP proteins that assemble during transcription of H/ACA RNA are in human (yeast): dyskerin (Cbf5p), hGar1p (Gar1p), hNhp2p (Nhp2p), and hNop10p (Nop10p) where dyskerin is the PUS catalyzing the reaction and is the only human RNA-dependent PUS (Li et al., 2016a). This assembly in human cells includes binding of dyskerin to hNop10, which binds to hNhp2 to

constitute the heterotrimer core of the complex. Dyskerin binds also Naf1, a chaperone present in early H/ACA RNP assembly, eventually exchanged with hGar1 for mature RNP formation (Darzacq et al., 2006; Hamma and Ferre-D'Amare, 2010).

The group of PUSs proteins acting independent of guide RNA, alone modifies tRNAs, rRNAs and snRNAs through recognition of structural and sequence motifs in their RNA substrate, e.g. Pus7p in *S. cerevisiae* recognizes a 7 nt long sequence and a stem-loop structure close to the target U nucleotide (Urban et al., 2009). Twelve RNA independent PUSs identified in human belong to five of six known PUSs families, named after prominent representative: tRNA uridine (Tru)A, TruB, TruD, RIuA and Pus10p, where RsuA-related family is missing compared to Eubacteria (Li et al., 2016a). They share some structural feature and require an aspartate in active site for catalysis, implying a common mechanism of action (Hamma and Ferre-D'Amare, 2006).

Genetic experiments revealed that several Pus proteins and/or snoRNAs are responsible for Ψ formation within mRNAs (Carlile et al., 2015; Lovejoy et al., 2014; Schwartz et al., 2014a).

4.1.3.1.4. Erasers and readers

Specific readers and erasers of Ψ have not been described yet.

4.1.3.1.5. Role in RNA function and fate

Studies have shown that the number of Ψ modifications increases with complexity of the organism (Ofengand and Bakin, 1997). Ψ formation is dynamically induced in response to environmental stimuli. However, the introduction of Ψ might not be reversible as it forms an inert C-C bond. Ψ thanks to an additional H-bond donor in the N1H binds a water molecule to bridge the interactions of this N1H and the preceding phosphate groups, thus promoting base stacking and hydrogen-bonding interactions that stabilizes RNA secondary and tertiary structures. It has previously been noted that Ψ sites in rRNA and snRNAs tend to occur in “important” regions that directly interact with other molecules. In U1, U2, U4, and U6 snRNAs, for example, Ψ sites occur at or close to a site that base-pairs with intronic RNA to facilitate RNA splicing (Charette and Gray, 2000). Mutations of yeast U2 snRNA at position 35 result in decreased splicing efficiency (McPheeters and Abelson, 1992). Ψ at this position was found to structurally stabilize an altered structure making the 2′-hydroxyl (OH) of the branch site accessible at the 5′ splice site thus facilitating RNA splicing (Newby and Greenbaum, 2001). In rRNA, there is a high density of conserved Ψ sites at

the PTC and at the decoding center of the 23S rRNA subunit, which interacts with mRNA and with the tRNA stem loop (Bakin et al., 1994; Lane et al., 1992).

Presence of a snoRNA in yeast that introduces highly conserved 2258 and 2260 Ψ residues located near the PTC of the ribosome of helix 69 in domain IV of the large subunit (LSU) rRNA provides a growth advantage (Badis et al., 2003). Furthermore, loss of Ψs in helix 69 impairs growth and affects ribosome synthesis indicating the relevance of these modifications for ribosome stability or assembly (Liang et al., 2009). In yeast, depletion of 1-5 snoRNA that guide formation of 6 Ψ in the PTC of rRNA suggested that inhibition of the formation of individual residues in yeast ribosomal RNAs depending on its location had no or mild effects on translation, while the lack of more residues has a cumulative effect on survival. Most probably a combination of modifications is required for proper structural formation and optimal functioning of ribosomes that ultimately can support cell survival (King et al., 2003).

In tRNA, Ψ at position 39 stabilizes its tertiary structure and through structural effects influences anticodon recognition (Yarian et al., 1999).

Besides the constitutive Ψ sites, stress-induced introduction of Ψ in U2 snRNA, U6 snRNA and mRNA has been reported. In *S. cerevisiae,* U2 snRNA has three constitutive Ψ sites at positions 35, 42 and 44. Under nutrient deprivation Ψ is incorporated at positions 56 and 93 by Pus7p and snR81. Presence of Ψ93 reduces pre-mRNA splicing showing function of inducible pseudouridylation (Wu et al., 2011a). Under starvation conditions that results in filamentous growth of *S. cerevisiae,* Pus1p was found upregulated and catalyzing Ψ28 in U6 snRNA. Mutations in specific sites of U6 snRNA can result in robust pseudouridylation at U28 and change in pseudohyphal growth phenotype suggesting that inducible Ψ plays a role also in cell physiology (Basak and Query, 2014).

The role of Ψ in mRNA has been speculated to be stabilizing or altering mRNA metabolism (Carlile et al., 2014; Schwartz et al., 2014a). While reading of Ψ by translating machinery is not qualitatively affected, translation and resulting protein expression are increased (Kariko et al., 2008). Kariko (et al.) showed that High-performance liquid chromatography (HPLC)-purified pseudouridylated mRNAs do not trigger an immune response and are more stable compared with mRNAs containing only uridine. Similarly, they show in mice that synthethic Ψ-modified erythropoietin mRNAs generates 10–100 times more erythropoietin compared to synthetic U-containing mRNAs (Kariko et al., 2012). Although Ψ – containing mRNA in rabbit reticulocyte

lysate showed enhanced translation, in wheat germ and *E. coli* it inhibited translation (Kariko et al., 2008). Altogether these data suggest Ψ translation effects might be machinery dependent.
One vertebrate H/ACA snoRNA E1/U17 (yeast homolog snR30) as part of H/ACA snoRNP was identified to take part in cleavage of precursor 18S rRNA (Atzorn et al., 2004). This SnR30 in yeast was shown to be essential for cell viability (Bally et al., 1988).
Mutations in the catalytic domain of dyskerin required for rRNA pseudouridylation have been implicated in Hoyeraal-Hreidarsson syndrome, which is characterized by severe spectrum of pathologies including immunodeficiency, growth retardation, and microcephaly (Knight et al., 1999; Yaghmai et al., 2000).

4.1.3.2. *2-Thiouridine (S2U)*

4.1.3.2.1. Discovery and detection of S2U

Replacement of oxygen at C2 position of the uracil ring with a sulfur atom results in 2-thiouridine (S2U) (Figure 12). S2U is a naturally occurring modification mainly identified in the first (wobble) position of the anticodon in yeast and in human at the wobble position (U34) of mitochondrial tRNA(Lys), tRNA(Glu) and tRNA(Gln) (Umeda et al., 2005). In yeast S2U is mainly found as part of 5- methylcarboxymethyl-2-thiouridine (mcm5S2U) while in human is 5-taurinomethyl-2-thiouridine (tm5s2U) of mitochondrial tRNA(Lys), tRNA(Glu), and tRNA(Gln) at the wobble position. S2U units were found to facilitate Watson–Crick base pairing with adenosine and the wobble pairing with G in the third position of the codon thus contributing to translational regulation through codon–anticodon interactions (Johansson et al., 2008).

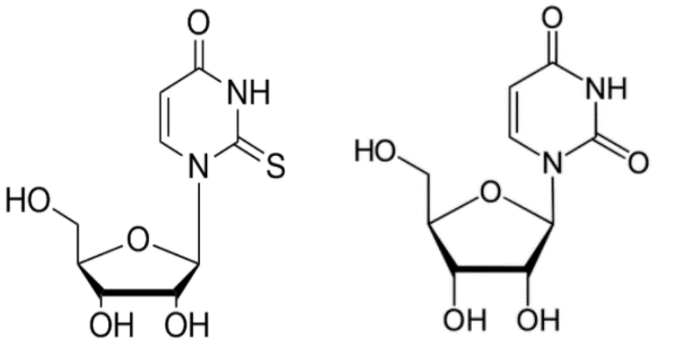

Figure 12. Structure of S2U (left) and uridine (right). Adapted from http://modomics.genesilico.pl/modifications/Y/.

4.1.3.2.2. Writers, erasers and readers

tRNA-modifying enzyme mitochondrial tRNA-specific 2-thiouridylase 1 (MTU1) is responsible for the 2-thiolation of the wobble position in human and yeast (mt)tRNAs. No specific erasers or readers have been identified.

4.1.3.2.3. Role of S2U in RNA function and fate

Disruption of the yeast MTU1 gene eliminates the 2-thio modification of (mt)tRNAs and impaires mitochondrial protein synthesis resulting in reduced respiratory activity (Umeda et al., 2005). S2Us are used together with other modifications by the innate immune system to distinguish between self and pathogen RNAs (Kariko et al., 2005).

4.2. RNA as a danger signal

Exogenous or endogenous RNA is a danger signal for the immune system (Rettig et al., 2010; Scheel et al., 2004; Tusup and Pascolo, 2017). Recognition of RNA is mediated through intracellular receptors located on the endosomal membrane or in the cytosol. Receptors associated with the endosomal membrane are members of the TLR family including: mouse and human TLR3 binding dsRNA (Alexopoulou et al., 2001), TLR7 (Diebold et al., 2004; Heil et al., 2004), human TLR8 recognizing single stranded RNA (Heil et al., 2004) and only present in mice TLR13 recognizing single stranded bacterial 23S rRNA (Oldenburg et al., 2012).

Expression of these receptors varies among cells. While TLR7 is predominately expressed in plasmacytoid dendritic cells (DCs) and, to some extent, in B cells and monocytes/macrophages (Gantier et al., 2008; Hornung et al., 2002), TLR8 is known to be primarily expressed in monocytes/macrophages and myeloid DCs (mDCs) (Hornung et al., 2002), TLR13 is mainly expressed in conventional mouse DCs and macrophages (Shi et al., 2011) and TLR3 is widely expressed in macrophages and mDCs but was also identified in fibroblasts, neurons, and epithelial cells (Cario and Podolsky, 2000; Hewson et al., 2005; Lafon et al., 2006; Matsumoto et al., 2002; Shi et al., 2000). Binding of RNA drives dimerization (TLR3 and TLR13) or conformational changes (TLR7 and TLR8) of these receptors resulting in their activation (Song et al., 2015). Upon activation these receptors recruit and interact with TIR-domain-containing adaptor proteins such as Myeloid differentiation primary response 88 (MyD88) (TLR7, TLR8 and TLR13) and TIR-domain-containing adapter-inducing interferon-β (TRIF) (TLR3).

In MyD88-dependent signaling, recruitment of a complex consisting in ubiquitin-ligases and kinases: TNF-associated factor 3 (TRAF3), TNF-associated factor 6 (TRAF6), IL-1R-associated kinase 1 (IRAK1), IL-1R-associated kinase 4 (IRAK4), inhibitor of NF-κB kinase (IKK)α, leads to activation and nuclear localization of interferon-regulatory factor 7 (IRF7), nuclear factor-κB

(NF- κB), activator protein 1 (AP-1) and cyclic AMP-responsive element-binding protein (CREB) where they trigger the production of type I interferon and pro-inflammatory cytokines.

TRIF interacts with receptor-interacting protein 1 (RIP-1), TRAF6 and TRAF3 which in turn interact with kinase complexes leading to activation of NF-κB, mitogen-activated protein kinase (MAPK) and interferon-regulatory factor 3 (IRF3) induction of inflammatory cytokines and interferon-beta (IFNβ) (Akira et al., 2006; Kawai and Akira, 2010).

Cytosolic receptors are part of retinoic acid–inducible gene (RIG)-I–like receptors (RLR) family where RIG-I and MDA5 bind dsRNA. Preferential binding to short dsRNA or 5′ppp uncapped ssRNA is attributed to RIG-I while MDA5 binds long, capped, di- or mono-5′ phosphate dsRNA (Hornung et al., 2006; Kato et al., 2008; Kato et al., 2006). Ligand binding induces RIG-I and MDA5 to assemble with mitochondrial antiviral-signaling protein (MAVS) on the mitochondrial and peroxisome membranes. Through caspase activation and recruitment domains (CARD)-CARD interaction MAVS undergoes activation thereby orchestrating recruitment of kinases IKKα, IKKβ and IKKγ that activate transcription factor NF-κB, and IKKε and TANK-binding kinase 1 (TBK1) activating IRF3 and IRF7. These transcription factors translocate to the nucleus and bind regulatory elements where they stimulate expression of type I interferon genes (Chan and Gack, 2016).

4.2.1. Immune recognition impaired by RNA modifications

Immunogenicity of RNA confines to their RNA modifications. Presence and abundance of RNA modifications correlates with the complexity of the organism. Studies have shown that total bacterial RNA is highly immunogenic for cytokine production in comparison with not immunostimulating total mammalian RNA in lipofectin-RNA transfected dendritic cells (Kariko et al., 2005).

The activation of immune receptors depends on the type and proportion of specific modifications in total RNA. Ivt RNAs activate human TLR3, TLR7 and TLR8, while chemically synthesized oligoribonucleotides (ORNs) stimulate murine TLR7 and human TLR8. Some sequences are known to enhance the potential of RNA to stimulate TLR7 and TLR8, such as GU- rich sequences and oligo-G tails (Heil et al., 2004; Hornung et al., 2005; Peng et al., 2005). In different cell types, such as HEK cells transfected with TLR3, TLR7 or TLR8 and monocyte-derived dendritic cells (MDDCs), transfection with lipofectin-ivt RNA containing m6A, m5C, Ψ, S2U or a combination

of modifications (m6A and Ψ) gave less or no immunostimulation in comparison to unmodified ivt RNA as recorded by production of interleukin (IL)-8, IL-12p70, tumor necrosis factor-alpha (TNF-α) and interferon-alpha (IFN-α). Presence of S2U and m6A in ivt RNA was shown to suppress recognition by TLR3 while m6A, m5C, Ψ and S2U limit the capacity of RNA to activate TLR7 and TLR8 in HEK cells transfected with respective TLRs. However, Ψ-containing ivt RNA was shown to activate TLR3 and induce IL-8, although to a lesser extent compared to unmodified RNA. This has been associated with base stacking properties of Ψ and possible stabilization of existing RNA duplexes. Conversely, presence of m6A destabilizes RNA duplexes thus preventing TLR3 activation (Kariko et al., 2005).

Polyinosinic:polycytidilic acid (poly(I:C)) is known to be a more potent inducer of IFN-α production compared to polyadenylic:polyuridylic acid (poly(A:U)) through TLR3 activation (Field et al., 1967). Introducing 2′-O-methylation in double-stranded RNA to generate poly(I:Cm) and poly(A:Um) reduced or abrogated triggering of IFN-α (De Clercq et al., 1972). 2′-O-methylation of uridines in single stranded ORNs blocks TNF-α induction in peripheral blood mononuclear cells (PBMCs) (Sioud, 2006). Recently it has been reported that 2′-O-methylation in ORNs turns a TLR7 agonist into a TLR8 specific ligand (Jung et al., 2015). Moreover, the 5′ cap structures of higher eukaryote mRNAs are known to possess ribose 2′-O-methylation in cap1 and cap2. Studies suggest that viruses incorporate similar or identical modifications in their mRNA to mimic host mRNA and evade immune system recognition. Sensing of 2′-O-methylation in viral RNA was attributed to MDA5 and TLR7 since infection of murine herpes virus (MHV) with mutated 2′-O-methyltransferase did not affect replication and spreading of viral mutants in MDA5 ko and TLR7 ko while wild-type mice were virus-free (Zust et al., 2011). Additionally, N1 2′-O-methylation in mRNA was shown to prevent binding to RIG-I thereby blocking its activation (Devarkar et al., 2016). Similarly, m6A and Ψ in polyU/UC RNA (derived from Hepatitis C virus genomic RNA) were identified to impede activation of RIG-I. However, these RNAs bound with poor (m6A RNA) and high affinity (Ψ) to RIG-I, but failed to trigger conformational changes in RIG-I required for its activation (Durbin et al., 2016).

SsRNA containing inosine and formulated in DOTAP (liposome) was shown to induce higher TNF-α production in PBMCs than a control RNA containing canonical nucleotides. Increase in TNF-production is mostly TLR8 dependent in human PBMCs. Moreover, this inosine ssRNA

induced lower IFN-α induction than unmodified RNA in human immune cells (Sarvestani et al., 2014).

Importantly, RNA immunostimulation studies have shown that 1.7-3.2% (14-29 nucleotides in 1571 long RNA) modified nucleotides are sufficient to decrease TNF-α to half of the amount induced by lipofectin- unmodified RNA transfection in MDDCs (Kariko et al., 2005).

Taken altogether, our immune system has evolved to distinguish heavily modified self RNA from less modified non-self RNA as a danger signal.

4.2.2. DNA-demethylating chemotherapies inducing endogenous RNA

DNA methylation inhibitors (DNMTis) were shown to be clinically efficient in solid and hematopoietic tumors. Their efficacy is attributed to demethylation of hypermethylated CpG island (CGI) present in the promoters of tumor-suppressor genes (TSGs) in cancer. However, recently a novel concept has emerged of possible DNMTis off-targets contributing to their potency. 5-aza-2-deoxycytidine (5-AZA-CdR) is a DNMTis used in hematological malignancies known to act as a cytidine analog that traps DNA methyltransferases after incorporation into DNA, resulting in global DNA demethylation as cells divide (Juttermann et al., 1994; Kelly et al., 2010). Transient low dose exposure to 5-AZA-CdR was shown to target colorectal cancer initiating cells by inducing dsRNA expression, activation of the cytosolic pattern recognition receptor MDA5 and downstream activation of MAVS and IRF7. Induced dsRNAs were identified as human endogenous retrovirus (ERV), reported to trigger signaling by cytosolic pattern-recognition receptors and activate MAVS in mammals (Roulois et al., 2015). Moreover, transient treatment of ovarian cancer cell lines with 5-azacytidine (Aza) and 5-aza-2′-deoxycytidine (Dac) shown upregulation in immune genes. These DNMTis trigger cytosolic sensing of ERV, with MAVS and TLR3 involved in Aza induced Type I Interferon response (Chiappinelli et al., 2015). Re-evaluation of known chemotherapies at low-doses and possibly in combination with other therapies could give insight into their immunomodulatory mechanisms and potentiate their anti-cancer efficacy.

4.3. Enhancing *in vitro* transcribed mRNA translation using RNA modifications

Therapeutic ivt mRNA production demands accurate structural mimicking of mature eukaryotic mRNA (emRNA). Similarly, to emRNA, ivt mRNA contains a 5′ cap, an open reading frame with start and stop codons and flanking 5′ and 3′ UTR sequences, and a 3′ poly(A) tail. For each

of these mRNA units, strategies are being developed to improve ivt mRNA stability and prevent immune recognition that would ultimately enhance its translatability in eukaryotic cells.

Current strategies include employment of:

- 5′ cap - Phosphorothioate modified- cap analogues capable of attracting eukaryotic translation machinery and preventing decapping enzymes responsible for mRNA decay (Kuhn et al., 2011). N1-2′-O-methylation to mimic Cap1 and Cap2 structures and prevent TLR7, MDA5 and RIG-I signaling (Grudzien-Nogalska et al., 2007; Kowalska et al., 2008). Introduction of base and ribose modifications – N1-methyl Ψ, Ψ, m5C, S2U, 5-methyluridine or m6A decreasing TLR3, TLR7, TLR8, RIG-I and protein kinase RNA-activated (PKR) signaling (Kariko et al., 2005; Kariko et al., 2008).
- Codon optimization – employment of frequent synonymous codons and CUG start codon instead of AUG (Gustafsson et al., 2004; Malarkannan et al., 1999).
- 3′ end of the poly(A) tail should not be masked by additional bases and that the optimal length of the poly(A) tail is between 120 and 150 nucleotides (Holtkamp et al., 2006).
- Incorporate 5′- and 3′-UTRs containing regulatory sequence elements that have been identified to modulate the translation and stability of endogenous mRNA such as 3′-UTRs of α- and β-globin mRNAs (Carralot et al., 2004; Holtkamp et al., 2006; Kariko et al., 1999).

Considering the technological advances improving quantification and localization of mRNA modifications, novel options could be exploited for enhancing ivt mRNA therapeutic properties (Tusup et al. 2017, *submitted*).

5. Aims of the thesis

Studies conducted in this thesis used formulations of RNA, with the following specific aims:

A1. Characterization of immunostimulatory profiles triggered by modified (natural) RNA molecules *in vitro*.

A2. Changing immunostimulation profiles of natural RNA by drug treatment steering RNA modifications.

6. Publications and manuscripts

Epitranscriptomics of cancer

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Keywords: RNA modifications, m6A, m5C, 2′O-mN, pseudouridine and inosine

Abstract

The functional impact of modifications of cellular RNAs, including mRNAs, miRNAs and lncRNAs, is a field of intense study. The role of such modifications in cancer has started to be elucidated. Diverse and sometimes opposite effects of RNA modifications have been reported. Some RNA modifications promote, while others decrease the growth and invasiveness of cancer. The present manuscript reviews the current knowledge on the potential impacts of N6-Methyladenosine, Pseudouridine, Inosine, 2'O-Methylation or Methylcytidine in cancer's RNA. It also highlights the remaining questions and provides hints on research avenues and potential therapeutic applications, whereby modulating dynamic RNA modifications may be a new method to treat cancer.

Key words: RNA modifications; m6A; m5C; 2'O-mN; Pseudouridine; Inosine

Core tip: The present manuscript reviews the current knowledge on RNA modifications in cancer. The potential impacts of N6-Methyladenosine, Pseudouridine, Inosine, 2'O-Methylation or Methylcytidine in cancer's RNA is presented and discussed. The review also highlights the remaining questions and provides hints on research avenues and potential therapeutic applications, whereby modulating dynamic RNA modifications may be a new method to treat cancer.

Tusup M, Kundig T, Pascolo S. Epitranscriptomic of cancer.

INTRODUCTION

Diverse and abundant modifications are introduced posttrancriptionally in cellular RNAs during their maturation. These modifications are made on canonical A, C, G, and U residues, and their formation is catalyzed by numerous specific enzymes or RNA-protein complexes (RNPs). Ribonucleotide residues can bear single or multiple modifications on the purine/pyrimidine ring and/or ribose. To date, over one hundred RNA modifications have been identified and listed in dedicated databases (http://mods.rna.albany.edu/; http://modomics.genesilico.pl)[1,2]. These naturally occurring modified nucleosides play various structural and functional roles in different types of RNAs: Transfer RNAs (tRNAs), ribosomal RNAs (rRNAs), messenger RNAs (mRNAs), small nuclear RNAs (snRNAs), microRNAs (miRNAs) and long non-coding RNAs (lncRNAs). The most widespread RNA modifications are base or ribose methylations, deamination of adenosine to inosine and isomerization of uridine into pseudouridine. Over the past decades these modifications have been studied in the context of malignancies. Frequently, a modification is found to have pro-cancer or anti-cancer effects depending on the type of RNA, the location of the modification and, most importantly, the cell type and context (*e.g.*, hypoxia). This review presents the current knowledge on the potential link between RNA modifications and cancer by systematically addressing the "pro-cancer", "anti-cancer" and mixed effects of RNA modifications. Since such a relationship has been reported for only some abundant modifications and for modifications for which a detection method is available [N6-Methyladenosine (m6A), 5-Methylcytidine (m5C), 2'O-mN, Ψ and I], the present review will focus on these modifications (Figure 1).

M6A IN CANCER RNA

Serendipitously discovered during the characterization of the mRNA 5' cap, methylation of the exocyclic nitrogen of adenosine, named m6A, is by far the most abundant mRNA modification, occurring on an average of three sites per mRNA[3-5]. Recent technological advances have facilitated m6A profiling across eukaryotes, including humans, mice[6],

yeasts[7], and plants[8,9], indicating that m6A is a conserved but dynamic modification. m6A has also been identified in rRNA[10], tRNA[11], snRNA[12], miRNA[13] and lncRNA[14].

M6A patterns are attributed to the consensus RRACH sequence (A is methylated; R = A or G; H = A, C, or U; and the first nucleotide next to m6A from the 5′ end most frequently is G), with preferential distribution near mRNA stop codons and 3′ untranslated regions (UTRs) and within long internal exons. Additionally, the m6A sites are conserved between human and mouse embryonic stem cells (ESCs) and somatic cells. However, distinct m6A patterns can also be detected among different species or cells at different developmental stages[4,7,15,16]. Some m6A signatures are tissue specific[4], and are altered in response to different stimuli[17], pointing to the potential role of m6A in regulating diverse cellular processes. m6A dynamics are assigned to the complex m6A enzymatic machineries, comprising m6A "writers", "readers" and "erasers". Although a plethora of studies suggest crucial and versatile roles of m6A and its machineries, its roles in cancer that have recently emerged are contradictory and require further investigation.

High m6A levels in cancers

"Writers" is a term given to enzymes that are part of the methyltransferase complex that introduces m6A. Components of this complex are methyltransferase-like 3 (METTL3)[18], METTL14[19], Wilms tumor 1-associated protein (WTAP)[20] and KIAA1429[21].

METTL3 protein levels were found to be elevated in lung adenocarcinoma cell lines compared to healthy tissue[22]. Depletion of METTL3 was shown to result in the inhibition of cancer cell growth, decreased invasive ability of cancer cells and increased cell apoptosis in the same study. Additionally, METTL3 was shown to function as an m6A-binding protein ('reader') in a specific subset of m6A-modified mRNAs, where it recruits eIF3 during translation initiation and therefore promotes translation. Expression of several oncogenes, including the mRNA of epidermal growth factor receptor (EGFR) and the Hippo pathway effector transcriptional co-activator with the PDZ-binding motif (TAZ) protein, was found to be promoted upon METTL3 recognition[22].

Similarly, in acute myeloid leukemia (AML), mRNA levels of METTL3 and METTL14 are significantly higher than in most cancers[23]. METTL3 depletion in MOLM13 caused differentiation and increased apoptosis, suggesting that high m6A levels may play a role in sustaining undifferentiated leukemic cells in AML[23] (Table 1).

Low m6A levels in cancers

Two m6A 'erasers' have been described: demethylases fat mass and obesity-associated protein (FTO) and alkB homolog 5 (ALKBH5)[24,25].

Single nucleotide polymorphisms within FTO known to be involved in the development of obesity in genome-wide association studies have been associated with the risk of developing diverse cancer types: Lung cancer, kidney cancer, high-grade prostate cancer, endometrial cancer, pancreatic cancer, pancreatic cancer in patients with type 2 diabetes, and breast cancer[26-33]. All these cancer types share a single SNP (rsrs9939609): The obesity-associated SNP in intron 1 of the *FTO* gene. This SNP was shown to increase primary transcript levels of the *FTO* gene, suggesting a gain-of-function mutation in cancers associated with this SNP[34].

In human epidermal growth factor receptor type 2 (HER2)-overexpressing subtypes of breast cancer, FTO is highly expressed in comparison to other breast cancer subtypes[35]. Contrary to the studies of high m6A levels in AML discussed in the previous chapter, low m6A levels have also been reported in AML subtypes. FTO expression can be upregulated by certain oncogenic proteins (*e.g.*, mixed lineage leukemia (MLL)-fusion proteins, promyelocytic leukemia/retinoic acid receptor alpha (PML-RARA), fms-related tyrosine kinase 3–internal tandem duplication (FLT3-ITD), and nucleophosmin 1 (NPM1) mutant), and dataset analysis of human AML confirmed that FTO was expressed at significantly high levels in t(11q23)/MLL-rearranged, t(15;17)/PML-RARA, FLT3-ITD, and/or NPM1-mutated AMLs[36]. Overexpression of FTO reduces m6A levels in ankyrin repeat and SOCS box containing 2 (ASB2) and retinoic acid receptor alpha (RARA) mRNA transcripts. It has been shown that the loss of m6A markings reduces mRNA stability, resulting in the partial repression of ASB2 and RARA expression in AML cells.

In four different AML cohorts, ASB2 and RARA exhibit a significant inverse correlation with FTO expression. ASB2 and RARA are upregulated during normal hematopoiesis and are important regulators of all-trans-retinoic acid (ATRA)-induced differentiation of leukemia cells. Through regulating the expression of such targets, FTO inhibits ATRA-induced AML cell differentiation. Both gain- and loss-of-function studies of FTO in leukemic cell models showed an oncogenic role of FTO in these AML subtypes[36]. However, recent studies have suggested that FTO acts as a demethylase of N6-2'O-dimethyladenosine in mRNA 5' caps, having only minor effects on m6A[37]. Thus, the role of FTO in AML might be independent of m6A.

Recently, both FTO and ALKBH5 have been found to play similar roles in glioblastoma stem cells (GSCs) and their tumorigenesis[38]. These studies shed light on their crucial roles in the regulation of mRNA m6A levels for maintaining GSC growth, self-renewal, and tumor development. Enhanced growth and self-renewal of GSCs *in vitro* were detected upon the depletion of METTL3 or METTL14, resulting in reduced mRNA m6A levels, and promoted the ability of GSCs to form brain tumors *in vivo*. Accordingly, treatment with the FTO inhibitor MA2, the ethyl ester form of meclofenamic acid, increased mRNA m6A levels and suppressed GSC growth *in vitro* and GSC-initiated tumorigenesis, ultimately prolonging the survival of GSC-engrafted mice.

In a similar study, the authors checked the expression levels of m6A regulators in available datasets for glioblastoma multiforme (GBM) and discovered elevated expression of m6A demethylase ALKBH5 that correlated with poor clinical outcomes for GBM patients[39]. Stable knockdowns in cultured human GSCs showed that the loss of ALKBH5 decreases GSC proliferation and reduces the expression of the stemness markers Nestin, Sox2, Nanog, and Oct4, which are normally expressed in GSCs. In rescue experiments, wild-type, but not catalytically inactive, ALKBH5 recovers the phenotype, suggesting that it plays a role in stemness maintenance and that the proliferation of GSCs is solely based on demethylation activity. Moreover, these authors examined the expression of transcription factor FOXM1 (forkhead box m1), which is known to play a pivotal role in regulating GSC proliferation, self-renewal, and tumorigenicity, and found

that it depends on ALKBH5 demethylating activity. All these findings were based on m6A hyper erasing, which opens new possibilities for promising targeted treatments in glioblastoma (Table 1).

It has been reported that the hypoxia-inducible factors (HIFs) HIF-1α and HIF-2α activate *ALKBH5* gene transcription under hypoxic conditions in breast cancer cells, thus inducing m6A demethylation. This demethylation was shown to stabilize NANOG mRNA and promote the breast cancer stem cell (BCSC) phenotype. Depletion of ALKBH5 in hypoxic breast cancer cells was identified as an effective strategy to decrease NANOG expression and limit the presence of BCSCs *in vivo*[40] (Table 1).

Mixed role of m6A in cancer

The primary microRNA (pri-miRNA) junction region between the hairpin stem and the flanking single-stranded RNA was found to be abundant in m6A consensus motifs. The recognition of the junction regions is mediated by Dicer, followed by the recruitment of the ribonuclease Drosha (the microprocessor complex), which cleaves the RNA duplex to yield the premiRNA product. Depletion of HNRNPA2B1 (a nuclear "reader") or METTL3 knockdown in HEK293 and MDA-MB-231 cells resulted in a significant reduction in the expression levels of the mature forms of a number of m^6A-marked miRNAs. The tumor-suppressor miRNA let-7 was significantly reduced upon the depletion of METTL3 possibly due to diminished Dicer binding to pri-miRNAs, thus preventing the formation of mature miRNAs. However, these METTL3-depletion experiments also showed a decrease in the expression of onco-miRNAs, such as miR-221 and miR-222[13,41]. Taken together, the presence of m6A affects diverse pri-miRNA and mature miRNA subpopulations, but its relevance in the context of cancer still needs to be investigated.

2'O-METHYLATION IN CANCER RNA

Methylation of the 2'-hydroxyl group of ribose is one of the predominant internal modifications of rRNA and snRNA[10,42]. This modification is also found in tRNA and

mRNA, mostly at the first and second nucleotides in Cap1 and Cap2 structures, respectively.

Introducing 2'O-methylation on ribose is mediated by complexes of guide RNA and proteins named small nucleolar ribonucleoprotein (snoRNP) complexes or by methyltransferases: human cap1 and 2, 2'-*O*-ribose methyltransferase, hMTr1 and hMTr2[43-45]. snoRNP complexes consist of Fibrillarin (the catalytic component in humans, also known as Nop1p in yeast), Nol5a (Nop56p), Nop58 and Snu13 subunits[46-48], which are guided by C/D Box snoRNAs to the appropriate base[49-50].

High 2'O-methylation levels in cancer

Tumor suppressor p53 and Fibrillarin seem to be linked[51]. Knockdown of p53 in cellular models of breast and colon cancer resulted in the overexpression of Fibrillarin at both the mRNA and protein levels. It is suggested that tumorigenesis associated to mutated p53 promotes an increase in the methylation status of rRNAs, which alters their ribozyme activity, thus affecting their translation fidelity and rate. Through the methylation of rRNA, Fibrillarin stimulates the translation of cancer-promoting proteins:
(1) Insulin-like growth factor 1 receptor (IGF1R), which plays a role in tumor progression, cell survival, and the response to chemotherapy (reviewed by Pollak *et al*[52]); (2) c-Myc, a pleiotropic pro-oncogene (reviewed by Dang *et al* [53]); (3) Fibroblast growth factor 1/2 (FGF1/2), which is involved in epithelial-mesenchymal transition[54], and (4) Vascular endothelial growth factor A (VEGFA), which acts in tumoral angiogenesis[51,55].

Translation of these proteins relies on internal ribosome entry site (IRES) in the mRNA, which is a 5' cap-independent translation mechanism that may be used in specific conditions. The inhibition of rRNA methylation was shown to impair IRES translation initiation by perturbing the association of the 40S and 60S subunits[56]. Therefore, it is conceivable that enhanced ribosomal methylation increases the translation of IRES-containing mRNAs. Nevertheless, clinical analysis shows that a high level of Fibrillarin in primary breast tumors is associated with poor survival, independent of other biological

markers[51]. Elevated expression levels of Fibrillarin were previously reported in primary and metastatic prostate cancers and in squamous cell cervical carcinoma (Table 1)[57,58].

NOL5A gene was found to be overexpressed in Burkitt's lymphoma-associated c-Myc mutants[59], and human NOP58 mRNA levels were found to be elevated in metastatic melanoma lesions[60].

Low 2'O-methylation levels in cancer

Contrary to Fibrillarin's indirect promotion of IRES-driven translation, in MCF-7, a breast cancer cell line, Fibrillarin knockdown resulted in the accumulation of p53, possibly affecting the UTR of the p53 mRNA and increasing IRES-driven *de novo* synthesis[61]. These studies suggest a complex interplay between p53 and Fibrillarin, while IRES-dependent translation is not exclusively stimulated by increased rRNA methylation.

Mixed 2'O-methylation in cancer

SnoRNA expression profiles were investigated in endometrial, lung and prostate cancers, as well as in glioma and chronic lymphocytic leukemia. High-throughput screening of snoRNAs in cancerous versus normal tissues underlined their overexpression or underexpression as common molecular events in tumorigenesis, with the former being more pronounced than the latter[62-66]. Analysis of blood serum has shown the possibility of detecting snoRNAs in breast cancer patient samples and the associated upregulation of a specific snoRNA, U6, in active disease [67]. Therefore, profiling snoRNAs with their respective RNA 2'O methylation modification signatures might be used as a noninvasive biomarker in the diagnosis and prognosis of cancer.

PSEUDOURIDINE IN CANCER RNA

The fifth base, known as pseudouridine (Ψ)[68], is one of the most abundant nucleotide modifications present in all three life domains[2]. After its initial detection in rRNA and tRNA, pseudouridine was detected in mRNA, lncRNA, and snRNAs, such as U2 snRNA and snoRNA[69,70]. Introducing Ψ in eukaryotic RNA can be mediated through guide

RNA-dependent H/ACA BOX snoRNA pseudouridine synthases (PUSs) or guide RNA-independent PUSs. A recent review by Penzo *et al*[71] reports on the functional roles of pseudouridines and related human pathologies.

Only low Ψ levels have been reported in cancer tissues/cells; thus, this chapter will contain only a section titled "Low Ψ levels in cancer". Surprisingly, elevated levels of circulating Ψ have been measured in the body fluids of cancer patients, but its role and origin are not well defined, so this finding will not be further discussed here.

The highly conserved protein dyskerin is the human PUS that catalyzes the pseudouridylation of snoRNPs that assemble during the transcription of guide H/ACA RNA. Mutations in the *Dkc1* gene coding for dyskerin can be found in the X-linked form of dyskeratosis congenita (DC). DC is a rare, inherited disorder that is characterized by mucocutaneous abnormalities and bone marrow failure. DC can be inherited as an X-linked recessive, autosomal dominant or autosomal recessive disease[72]. Although the absence of dyskerin, which results in the loss of pseudouridine in rRNA, was suggested as a primary cause of DC, a recent study assigned telomerase dysfunction as the primary cause of DC[73]. Namely, mutations in H/ACA-resembling domains in the RNA component of telomerase RNP, which are required for telomerase accumulation, stability, 3' end processing and function, are associated with an autosomal form of DC[74-76] .

In patients with DC, a higher predisposition to cancer has been reported, although low mutational frequency in the *DKC1* gene was shown in primary tumors[77]. This predisposition might be a synergistic outcome of impaired pseudouridylation. Most likely, the dysregulation of rRNA pseudouridylation precedes disease onset, as studies in hypomorphic *Dkc1*-mutant mice suggest. A specific defect of the internal ribosome entry site also occurs upon DKC1 loss, causing a specific defect in the translation of some IRES-containing mRNAs. Ribosomes that lack pseudouridine modifications show a direct impairment in binding to IRES elements[78]. Consequently, in hypomorphic DKC-1 mice, cap-dependent translation of mRNA is not compromised, but translation of IRES-containing mRNAs, including the tumor suppressors p27 and p53, is perturbed[79-82], resulting in a higher incidence of cancer development in these mice. Thus, this impaired

translation of tumor suppressor mRNA might also be a driver of cancer in DC patients. Moreover, recent identification of Ψ in mRNA[83] brings an additional level of complexity and regulation of the expression of target RNAs.

In hematological cancers, such as leukemias, lymphoma and multiple myeloma, downregulation of specific subsets of dyskerin-associated H/ACA snoRNAs has been demonstrated[84-86] (Table 1). Thus, lower pseudouridylation levels are a widespread feature of cancer.

INOSINE IN CANCER RNA

Inosine is an RNA modification resulting from the hydrolytic deamination of adenosine catalyzed by adenosine deaminase enzymes acting on double-stranded RNA (ADAR) or adenosine deaminase acting on transfer RNA (ADAT), which are families known to function in A-to-I RNA editing. Enzymes of the ADAR family are catalytically active ADAR1, ADAR2 and ADAR3, which still has an unknown function.

ADARs introduce inosine in coding and non-coding RNAs and have drastic impacts on the cellular transcriptome and translatome. The hypo- or hyper-editome has been associated with diverse types of cancer. The role of ADAT in cancer has not been reported.

High editing levels in cancer

Most frequently editing locations are long, partially complementary RNAs formed from inverted non-coding repeats, such as *Arthrobacter* ***lu****teus* (Alu) and long interspersed **e**lement (LINE) located in mRNA UTRs and introns. Two major studies have investigated RNA-editing patterns in tumors versus normal tissues. Each of the studies employed RNA-Seq datasets from The Cancer Genome Atlas (TCGA) project (https://cancergenome.nih.gov/) and compared them to reference datasets of editing sites. High-confidence RNA editing sites are annotated in the Rigorously Annotated Database of A-to-I RNA Editing (RADAR, http://rnaedit.com/), where one study focused on detecting Alu and non-Alu RNA editing events in 17 cancers, whereas the other study focused on Alu RNA editing events in 9 different cancers[87,88].

In general, elevated Alu editing activity in tumors compared to matched normal tissues was found in bladder urothelial carcinoma (BLCA), breast invasive carcinoma (BRCA), colon adenocarcinoma (COAD), head and neck squamous cell carcinoma (HNSC), lung adenocarcinoma (LUAD) and thyroid carcinoma (THCA). This hyperediting of Alu was attributed to ADAR1, whose expression levels matched in all these types of cancer, except COAD. Similarly, a study by Han et al. where more patient samples and non-Alu edited sequences were included, confirmed hyperediting in BLCA, BRCA, HNSC, LUAD, THCA compared to normal tissues. Again, increased editing levels correlated with the mRNA levels of ADAR1.

Increased ADAR-1 levels were reported in non-small cell lung cancer (NSCLC), hepatocellular carcinoma (HCC), esophageal cell carcinoma (ESCC), gastric (GC) and cervical cancer, suggesting that tight regulation of editing levels might have implications in cancer development and that ADAR1 might act as an oncogene[89-92] (Table 1 and Figure 2).

Recoding editing: In non-small cell lung cancer samples, ADAR1 gene amplification was shown to increase the editing of the DNA base excision repair glycosylase enzyme NEI-like protein 1 (NEIL1). Pre-mRNA editing of NEIL-1 causes a lysine to arginine (K242R) change in the lesion recognition loop of the protein. The edited NEIL1 protein removes thymine glycol from duplex DNA at a lower rate compared to the unedited form, while repair of the guanidinohydantoin lesion is enhanced by edited NEIL1[93]. In overexpression experiments, transfection of edited NEIL1 enhanced the growth of A459 cells in comparison with the transfection of unedited transcripts. Thus, increased recoding editing of NEIL1 as a proposed target of ADAR1 could contribute to the phenotype of lung cancer cells[94].

AZIN1 (encoding antizyme inhibitor 1) is edited by ADAR1, which has increased expression levels in HCC and was found to positively correlate with AZIN1 editing frequency. AZIN1 is an antizyme inhibitor whose activity is crucial in limiting cellular proliferation. Antizyme binds and induces the degradation of the growth-promoting

proteins ornithine decarboxylase (ODC) and cyclin D1 (CCND1)[95,96]. AZIN1 is homologous to ODC and has a greater binding affinity to antizyme compared to ODC. Binding of AZIN1 to antizyme prevents the degradation of ODC[97]. Thereby, AZIN1 acts as an oncogene by inhibiting the tumor-suppressor activities of antizyme[96]. AZIN1 expression was found to be substantially elevated in cancers of the prostate, brain, breast and liver, and gene expression data have identified alterations in the AZIN1-to-antizyme ratio in many human cancers, confirming its role in promoting growth[98-100]. In HCC, increased A-I editing of the AZIN1 transcript introduces serine-to-glycine substitution at residue 367 in the protein. This recoding editing is associated with conformational changes and translocation from the nucleus to the cytoplasm and results in a higher-binding affinity to antizyme and greater protein stability, thus promoting cell proliferation. AZIN editing increases during the progression from primary liver cancer and cirrhosis to advanced HCC with recurrence and metastasis, suggesting its use as a prognostic marker[101] . It is plausible that similar editing events occur in other types of cancer, as has been confirmed in esophageal squamous cell carcinoma (ESCC) and breast cancer[87,92,102]. Recently, tumorigenesis of NSCLC was also attributed to high levels of AZIN1 editing[103]. It has been reported that AZIN1 editing levels correlate with sensitivity to drug treatment in cancer cell lines. Cancer cells lines with increased levels of AZIN1 editing showed more sensitivity to some of the chemotherapies used in small cell lung cancer (SCLC): paclitaxel, irinotecan, and topotecan[87].

Filamin B (FLNB) is an actin cross-linking protein and, together with filamin A, it forms homo- and heterodimers mediating orthogonal branching of actin filaments [104]. Filamin B is known to be a target for editing [105], and interestingly, one recoding editing event was shown to be increased in two types of cancer. In HCC, ADAR1 and ADAR2 were both reported to mediate FLNB transcript editing in codon 2269, resulting in the amino acid change Met→Val. Increased editing of FLNB compared to matched non-tumor liver tissues has been closely associated with HCC pathogenesis from normal to clinically verified HCC. In this study, ADAR1 levels were shown to be increased, while ADAR2 levels were decreased in HCC samples compared to non-tumor liver tissues[90]. The same

recoding editing was found in ESCC, where unlike HCC, only ADAR1 was responsible for this hyperediting. FLNB hyperediting correlated with ADAR1 levels in ESCC samples[92]. The functional role of FLNB editing is not known.

Ras homolog family member Q (RHOQ) belongs to a family of Rho GTPases that are known to be intracellular signaling molecules regulating the actin cytoskeleton and thereby cellular functions, such as cell polarity, migration, and vesicular trafficking. Rho GTPases are present as either an active GTP-bound form or an inactive GDP-bound form[106]. Activation of Rho GTPases is implicated in the development and progression of many types of human malignancies, including CRC[107]. RhoQ has been most extensively studied for its central role in insulin-stimulated GLUT4 transport in adipocytes[108]. Amino acid substitution of asparagine with serine (N136S) in the edited RhoQ was identified in colorectal cancer (CRC). The ADAR responsible has not been identified. This editing was suggested to change RhoQ protein–protein interactions and induce increased levels of RhoQ binding of GTP, causing actin cytoskeletal reorganization and increased invasion potential without affecting proliferation in CRC cell lines. Moreover, edited RHOQ was associated with recurrence of CRC when present in the tumor[109].

Protein tyrosine phosphatase non-receptor type 6 (PTPN6) is a cytoplasmic protein expressed in hematopoietic cell development, proliferation and the receptor-mediated mitogenic signaling pathway[110,111]. In bone marrow mononuclear cells (BMMCs) of patients with acute myeloid leukemia, a novel PTPN6 transcript retaining intron 3 has been identified. This transcript arises from an alternative splicing reaction where editing-mediated deamination of A7866 in intron 3 erases this branch formation site, making it invisible to the splicing machinery. The ADAR responsible has not been identified. It is suggested that this retention results in the translation of a nonfunctional protein where the intron 3-encoded sequence is located in the N-terminal Src homology 2 (SH2) domain. PTPN6 binding with partner proteins, such as proto-oncogene receptor tyrosine kinase - c-Kit[112], and its self-inhibition of phosphatase activity occurs via its N terminal domain[113]. All this information suggests that its deregulation ultimately leads to uncontrolled hematopoietic growth and function. The tumor-specific editing seen in

AML might correlate with the clinical course of the disease since low levels of intron-retaining transcripts in patient BMMCs at remission compared to those at diagnosis suggest that editing promotes tumorigenesis[114].

High miRNA editing levels in cancer: ADAR1 gene amplification in NSCLC demonstrated ADAR1 overexpression in patients with early-stage lung cancer, underlining its potential oncogenic role in this cancer. Increased levels of ADAR1 corresponded with edited miR-381 levels in NSCLC. Overexpression of edited miR-381 in NSCLC possibly contributes to stemness and chemoresistance[94].

Low editing levels in cancer

Low levels of mRNA open reading frame editing in cancer: The same correlation but in the opposite direction was shown in hypoedited cancers, such as kidney chromophobe (KICH) and kidney renal papillary cell carcinoma (KIRP), with ADAR1 mRNA paired editing levels. The ADAR2 levels checked in both studies showed a complex expression pattern but no matching editing levels (Table 1 and Figure 2)[87,88].

The role of ADAR1 in breast cancer is not fully understood. A recent study reported high ADAR1 expression in half of the examined triple-negative-cancer patients[115]. Conversely, it has been proposed that ADAR1 prevents tumor progression by editing the transcript coding for the alpha-3 subunit of gamma-aminobutyric acid type A (Gabra3).

The chloride-permeable gamma-aminobutyric acid type A ($GABA_A$) receptors are crucial mediators of fast inhibitory neurotransmission in the central nervous system[116]. The Gabra3 transcript undergoes recoding editing of isoleucine to methionine (I/M) in the third transmembrane region. This substitution was found to affect $GABA_A$ surface presentation and its cellular trafficking[117]. In addition to being normally expressed in normal neuronal tissues, Gabra3 has been identified in breast cancer, where its high expression inversely correlates with breast cancer survival. ADAR1-edited Gabra3 was found in non-invasive breast cancer cell lines and was linked with the protein kinase B (Akt) pathway. A proposed mechanism for the non-invasive phenotype is that Gabra3

editing reduces its surface expression and indirectly prevents Akt activation, thereby preventing cell proliferation and invasiveness. Thus, the unedited form of Gabra3 in breast cancer is suggested to promote tumor progression, invasion and metastatic potential[118].

Lower ADAR2 levels are recognized in gastric cancer, glioblastoma, HCC and ESCC [91,119-120] (Figure 2). ADAR2 levels were found to correlate with changes in podocalyxin-like (PODXL) and GluR-B functions. The PODXL RNA editing event is an amino acid substitution from histidine (His) to arginine (Arg) at codon 241. This editing in the gastric cancer cell line MKN28 was shown to prevent increased growth rates and invasive capability compared to cells with the unedited form. Moreover, recoding editing of a single position located in the channel-pore-loop domain in GluR subunit B (GluR-B) (the Q/R-site) from Gln to Arg results in a channel that is impermeable to Ca^{2+}[121]. Tight regulation of editing is essential for the adequate function of this channel. Hypoedited PODXL and GluR-B with altered functions are associated with gastric cancer and malignant glioblastoma, respectively. Consequently, a tumor-suppressor role has been attributed to ADAR2[91,119].

In ESCC, it has been reported that ADAR2 promotes apoptosis by editing and stabilizing insulin-like growth factor-binding protein 7 (IGFBP7) RNA. IGFBP7 is a secreted factor binding to and interfering with the activation of IGF1R. Through receptor occupation, IGFBP7 blocks downstream phosphatidylinositol 3-kinase (PI3K)-AKT signaling, resulting in the inhibition of protein synthesis and cell apoptosis[122]. IGFBP7 was previously reported to be an apoptotic promoter in prostate cancer[123], colorectal cancer[124] and breast cancer[125]. The editing site in IGFBP7 is at position 284 of the coding sequence, and codon 95 is changed from AAG (lysine) to AIG, which is read as AGG (arginine) (K95R). This editing was shown to protect IGFBP7 against matriptase proteolysis in ESCC culture and xenografts, thus enabling the proapoptotic function of IGFBP7. ADAR2 is known to be downregulated in ESCC, and its upregulation induces apoptosis in ESCC cell lines *in vitro*, suggesting that IGFBP7 underediting may promote tumorigenesis in esophageal squamous cell carcinoma[120].

Low miRNA editing levels in cancer: ADAR2 rescue in glioblastoma cells was shown to inhibit cell proliferation and migration, confirming its possible tumor-suppressor role [126]. This anti-tumor effect might be explained though the regulation of onco-miRNAs in glioblastoma. Three particularly investigated onco-miRNAs, miR-221, miR-222 and miR-21, are overexpressed in glioblastoma[127]. ADAR2 can edit miR-222/221 and miR-21 precursors and decrease the expression of the corresponding mature onco-miRNAs in the normal mouse brain and in different lines. Decreased levels of ADAR2 identified in glioblastoma probably push the balance of onco-miRNA/tumor-suppressor miRNA towards increased expression of onco-miRNAs, such as miR-221, miR-222 and miR-21, thereby supporting tumor progression[128].

In the human brain, the miR-376 cluster encodes 4 pri-miRs that give rise to 5 distinct mature miRNAs, which are subjected to specific A-to-I RNA editing on 9 adenosines. In noninvasive U87 glioma cells, the expression of the unedited miR-376a* was shown to promote aggressive tumor migration and invasion of these cells both *in vitr*o and *in vivo*. The editing reaction missing in the GBM cell lines generally occurs in the seed region of pri-miR-376a1 at the +9 site, ultimately giving rise to mature edited miR-376a*. The absence of this editing changes the specific targets of the miRNA. It has been identified that nonedited miR-376a*, through its binding to 3′ UTR, has a novel target, RAP2A, which is a member of the RAS oncogene family with an unknown function. However, the nonedited miR-376a* targeting of RAP2A is unable to target the autocrine motility factor receptor (AMFR), resulting in its upregulation and possibly contributing to increased migration and invasiveness of glioma cells[129].

Melanoma is the most aggressive type of skin cancer. It has been reported that there is a significant decrease in ADAR1 expression in ~65% of metastatic melanoma specimens compared to melanocytes[130] (Table 1 and Figure 2). ADAR-1 transcripts were found to be targeted by miR-17 and miR-432, thus decreasing ADAR1 expression. Both miR-17 and miR-432 were identified to be overexpressed in melanoma possibly due to the amplification of encoding genes[130]. However, studies suggest that ADAR1

insufficiencies contribute to the enhancement of proliferation of melanoma cells through editing the independent regulation of miRNA biogenesis. miRNA-455-5p was identified as a target of ADAR1 in low-metastatic melanoma cells but not in highly metastatic cell lines. ADAR1 was shown to edit pri-miR-455-5p at +2 and +17 positions. This editing probably results in the reduction of the processing of pri-miRNA by Dicer or Drosha by lowering the binding affinity. However, it is also possible that ADAR1 binds to Dicer since the amount of miR-455-5p bound to Dicer and Drosha was inversely correlated with ADAR1 expression. ADAR1 was shown to form a complex with Dicer through protein-protein interactions[131]. In this study, the authors gave a model of RNA editing in the context of melanoma progression and metastasis, where cAMP response element binding (CREB) downregulates ADAR1 and gives rise to non-edited miR-455-5p. Expression of miR-455-5p suppresses the tumor suppressor gene cytoplasmic polyadenylation element-binding protein 1 (CPEB1), resulting in growth promotion and metastasis in melanoma cells[132].

M5C IN CANCER RNA

Cytosine base methylation - m5C has been identified in rRNA, tRNA and recently in mRNAs and is particularly enriched in untranslated regions and near Argonaute-binding regions[133]. The enzymes responsible for the introduction of m5C are members of the DNA methyltransferase homolog (Dnmt2) and the NOP2/Sun (NSun 2 and 4) RNA methyltransferase family[134-136]. The role of these enzymes in the methylating activities of tumorigenesis is currently unknown. However, in circulating tumor cells from lung cancer patients, increased RNA m5C levels were shown compared to those in whole blood cells[137]. Further investigation of the role of m5C in cancer is required.

Concluding remarks

Tight regulation of the writing, reading and eventual erasing of RNA modifications is essential for RNA metabolism. Misbalanced expression of the enzymes responsible for introducing, and in some cases removing, these modifications is considered a possible

signature for specific types of cancer (Table 1). Considering the broad effect of RNA modifications on tumor cell biology, future methylome, pseudome and editome studies will shed light on those relatively unexplored epitranscriptomic mechanisms in tumors. Those studies will pave the way for the development of anti-cancer drugs that could act by steering RNA modifications.

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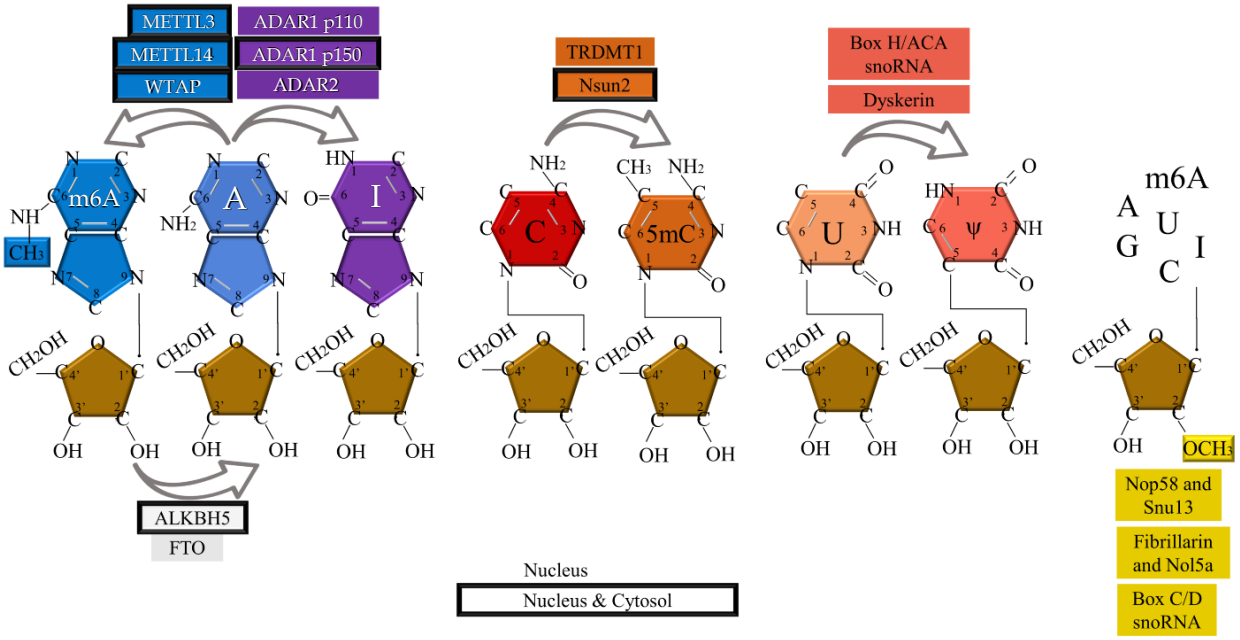

Figure 1. Ribonucleotide RNA modifications known to be of relevance in cancer and their enzymatic machineries. m6A: N6-methyladenosine; METTL3: Methyltransferase like 3, METTL14: Methyltransferase like 14, WTAP: Wilms' tumor 1-associating protein; m6A erasers: ALKBH5: Alkylation repair homologue protein 5, FTO: Fat mass and obesity-associated protein. Inosine (I) writers: ADAR1 (p110 and p150) and ADAR2: Adenosine deaminase acting on RNA 1 and 2. 5-methylcytosine (5mC): NSUN2: NOP2/Sun domain protein 2, TRDMT1: tRNA aspartic acid MTase 1. 2'O-methylation writers: Nol5a: Nucleolar Protein 5A, Nop58: Nucleolar protein 58, Snu13: Small Nuclear Ribonucleoprotein 13, SnoRNA: Small nucleolar RNA.

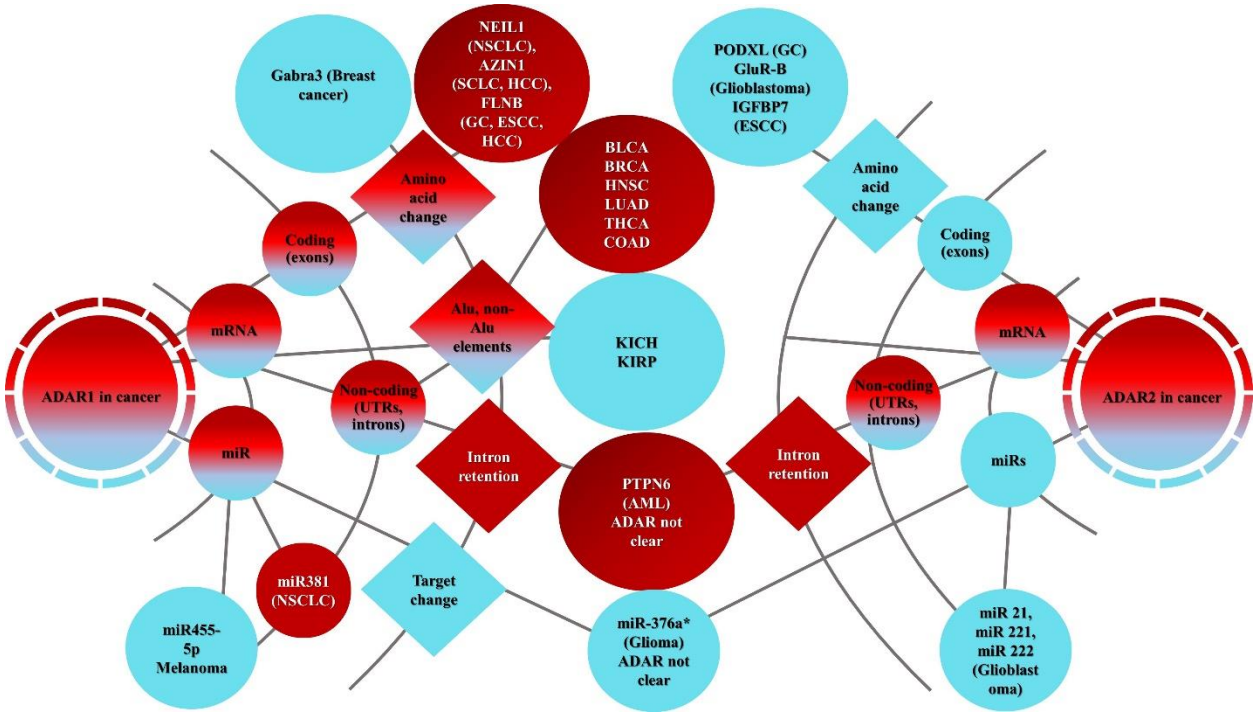

Figure 2 Editing of double-stranded RNA on mRNA and miRNA. Below, high ADAR1 levels are associated with breast cancer, NSCLC, colon cancer and cervical cancer, while low ADAR1 levels are shown in melanoma. Low levels of ADAR2 are present in glioblastoma. HCC, ESCC and gastric cancer are indicated as ADAR2-low and ADAR1-high cancers. The potential roles of ADAR1 and ADAR2 in cancer are depicted by mind map. Red means high expression/inosine content and blue means low expression/inosine content. In circles are the name of molecules/cancers and in diamonds are the mechanisms. Abbreviations: NSCLC: non-small cell lung cancer, HCC: hepatocellular carcinoma, ESCC: esophageal cell carcinoma, GC: gastric cancer, ESCC: esophageal squamous cell carcinoma, AML: acute myeloid leukemia, SCLC: small cell lung cancer, BLCA: bladder urothelial carcinoma, BRCA: breast invasive carcinoma, COAD: colon adenocarcinoma, HNSC: head and neck squamous cell carcinoma, LUAD: lung adenocarcinoma, THCA: carcinoma, KICH: kidney chromophobe, KIRP: kidney renal papillary cell carcinoma, NEIL1: NEI-like protein 1, Gabra3: alpha-3 subunit of

gamma-aminobutyric acid type A, FlnB: Filamin B, PTPN6: Protein tyrosine phosphatase non-receptor type 6 , PODXL: podocalyxin-like, GluR-B: glutamate R -B, IGFBP7: insulin-like growth factor-binding protein 7 .

Table 1. Relative amount of modification (directly quantified or extrapolated from the expression level of writers/erasers). In brackets are the names of genes that have been analyzed

RNA modifications	High in cancer	Low in cancer
m6A	Lung adenocarcinoma[22], AML[23].	HER2 overexpressing subtypes breast cancer[35], t(11q23)/MLL-rearranged, t(15;17)/PML-RARA, FLT3-ITD, and/or NPM1-mutated AMLs (ASB2 and RARA)[36], GBM (FOXM1)[39], breast cancer (NANOG)[40].
2'O-Methylation	Breast cancer [51][67]; primary and metastatic prostate cancers[58], squamous cell cervical carcinoma[57].	
Ψ		Leukemia, lymphoma, multiple myeloma[84-86]
Inosine	BLCA, BRCA, COAD, HNSC, LUAD, THCA[87-88], NSCLC (NEIL1[94], AZIN1[103]), miR-381[94]), SCLC	KIRP, KICH[87-88], Breast cancer (Gabra3)[118], Gastric cancer (PODXL)[91], Glioblastoma (GluR-B)[119], onco miR-21, miR-221, miR-222[128], ESCC (IGFBP7)[120], Glioma (miR-376a*)[129], Melanoma[130] (miR-455-5p)[132].

	(AZIN1)[87], HCC (AZIN1 [101],FLNB[90]), GC[91], ESCC (FLNB)[92], cervical cancer[89], CRC (RHOQ)[109], AML (PTPN6)[114]
5mC	circulating tumor cells in lung cancer[137]

AZIN1: Antizyme inhibitor 1, RHOQ: Ras homolog family member Q , PODXL: Podocalyxin-like , IGFBP7: Insulin-like growth factor-binding protein 7 , PTPN6: Protein tyrosine phosphatase non-receptor type 6 , NEIL1: NEI-like protein 1 , GluR-B: Glutamate R-B, Gabra3: Alpha-3 subunit of gamma-aminobutyric acid type A , FlnB: Filamin B, ASB2: Ankyrin repeat and SOCS box containing 2 , RARA: Retinoic acid receptor alpha, FOXM1: Forkhead box protein M1, GBM: Glioblastoma multiforme , HER2: Human epidermal growth factor receptor type 2, MLL : Mixed lineage leukemia , PML/RARA: Promyelocytic leukemia/retinoic acid receptor alpha, FLT3-ITD: Fms-related tyrosine kinase 3–internal tandem duplication, NPM1: Nucleophosmin 1, NSCLC: Non-small cell lung cancer, HCC: Hepatocellular carcinoma, ESCC: Esophageal cell carcinoma, GC: Gastric cancer, CRC: Colorectal cancer , AML: Acute myeloid leukemia , SCLC: Small cell lung cancer, BLCA: Bladder urothelial carcinoma, BRCA: Breast invasive carcinoma, COAD: Colon adenocarcinoma, HNSC: Head and neck squamous cell carcinoma, LUAD: Lung adenocarcinoma , THCA: Thyroid carcinoma, KICH: Kidney chromophobe , KIRP: Kidney renal papillary cell carcinoma.

An eiF4G-recruiting aptamer increases the functionality of *in vitro* transcribed mRNA

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Abstract

As a versatile and safe vector, *in vitro* transcribed messenger RNA (ivt mRNA) is currently being intensively evaluated as an active pharmaceutical ingredient. Its therapeutic uses encompass vaccination, cell reprogramming, genome engineering, gene complementation and the expression of protein drugs (*e.g.*, growth factors or antibodies). The therapeutic efficacy of ivt mRNA correlates with the efficacy of its translation. Untranslated regions (UTRs) from stable mRNA, such as globin mRNA, and optimized 5' cap structures have been used to improve the functionality of ivt mRNA. However, the recruitment of the eukaryotic initiation factor 4E (eIF4E) protein to the 5' end of transfected ivt mRNA remains a rate-limiting parameter for translation. We added aptamer sequences that bind the eiF4G protein to the 5' UTR of ivt mRNA. One of these aptamer sequences produced a several fold increase in ivt mRNA expression (specifically, an increase of threefold to over tenfold depending on mRNA sequence and cell type). This simple modification of the 5' UTR of ivt mRNA may represent an efficacious and general method for improving the therapeutic index of all new mRNA-based therapeutic products.

1 Introduction

The expression of recombinant genes *in vivo* can be used for vaccination and gene therapy. Although plasmid DNA and recombinant viruses were initially used for this purpose, *in vitro* transcribed messenger RNA (ivt mRNA) emerged as a safe alternative in the 1990s and has become a broadly accepted method that has attracted increasing interest during the last ten years (Sahin et al., 2014). With the implementation of the first

good manufacturing practice (GMP) and large scale mRNA production facility (Pascolo, 2004; Probst et al., 2007), several human clinical studies demonstrating the safety, versatility and efficacy of directly injected ivt mRNA have been published (Bahl et al., 2017; Kranz et al., 2016a; Rittig et al., 2011, 2016; Sahin et al., 2017b; Weide et al., 2008; Weide et al., 2009). Key features of mRNA-based therapies are (i) the intrinsic translatability of the ivt mRNA (the level of protein expression),(ii) the ivt mRNA's immunogenicity (whether innate immune receptors are triggered) and (iii) formulation (site of delivery and expression). With respect to the former consideration, the optimization of the cap structure, untranslated regions (UTRs), open reading frames and poly A-tail has been reported (Holtkamp et al., 2006; Sahin et al., 2014). Due to such optimization, ivt mRNA has become a vector with high expression of the encoded protein. One limiting factor in cellular mRNA translation is eukaryotic initiation factor 4E (eIF4E), which recognizes the 5' cap structure of mRNA (Lee and Pelletier, 2012). eIF4E then recruits the scaffolding protein eIF4G, which, in turn, recruits all other components of the initiation complex to form the eIF4F complex that moves along the mRNA to the ATG start codon. We rationalized that direct recruitment of eIF4G to the 5' UTR could improve the efficacy of translation of ivt mRNA. Aptamers that bind eIF4G have been identified in attempts to block translation in tumor cells (Miyakawa et al., 2006). Miyakawa *et al*. demonstrated that three out of eight such aptamers indeed inhibited translation. We took advantage of the aptamers that did not block translation; in particular, we utilized these aptamers as bait to bring eIF4G to the 5' end of ivt mRNA. One of the non-blocking aptamers was most efficient at enhancing the translation of a downstream open reading frame. The addition

of this aptamer to ivt mRNA could be proposed as a universal method for improving ivt mRNA-based therapies.

2. Materials and Methods

2.1 Production of ivt mRNA

Plasmids containing a wild-type luciferase-coding gene or an optimized luciferase-coding gene (a synthetic gene purchased from Blue Heron Bio with a 5’ UTR of human alpha globin, a codon-optimized open reading frame and a double 3’ UTR from human beta globin) were used as matrices for PCR amplification with upstream primers that contained a T7 promoter followed by a sequence complementary to the 5’ end of the targeted gene, with or without an aptamer sequence between the promoter and the complementary sequence. Similarly, a synthetic codon-optimized gene encoding ZsGreen (a synthetic gene purchased from Blue Heron Bio with a 5’ UTR of human alpha globin, a codon-optimized open reading frame and a double 3’ UTR from human beta globin) was amplified using PCR. The primer sequences and corresponding mRNA products are presented in supplementary Figure 1. The PCR products were analyzed on an agarose gel and purified using a PCR cleanup kit from Qiagen in accordance with the manufacturer’s instructions. mRNA was produced by utilizing a HiScribe™ T7 ARCA mRNA Kit (with tailing) (New England Biolabs) to obtain ARCA-capped poly-adenylated mRNA or using GenScript capping and poly-adenylation kits on mRNA produced using a T7 RNA polymerase (New England Biolabs) reaction containing all 4 canonical

nucleotides or a mixture of A, C, G, and pseudouridine (as triphosphate nucleotides from TriLink). Capped poly-adenylated mRNA was precipitated using LiCl, washed with 75% ethanol, and resuspended in pure water. This mRNA was then quantified using a NanoDrop instrument and analyzed via agarose gel electrophoresis.

2.2 Cells and transfection

Human embryonic kidney (HEK) cells and CT26 mouse colon carcinoma cells were maintained in RPMI medium (Thermo Fisher Scientific) containing 10% fetal calf serum (FCS) and 0.2% antimicrobial reagent Normocin (Invivogen). Human dendritic cells were produced from adherent monocytes harvested from peripheral mononuclear blood cells (PBMCs) after 45 minutes of adhesion to plastic and cultured for 6 days in the presence of GM-CSF (800 U/ml) and IL4 (500 U/ml) from PeproTech (with medium replacement at day 3). They were eventually matured by 24 hours culture in the presence of Protamine-RNA nanoparticles at 5 μg/ml (Tusup and Pascolo, 2017). Transfections were performed with 200 000 cells per well in 100 microliters of RPMI medium supplemented with 10% FCS and 0.2% antimicrobial reagent Normocin (Invivogen) by adding either 200 ng of mRNA in 12 microliters of Opti-MEM (Thermo Fisher Scientific) and 200 ng of Lipofectamine 2000 (Thermo Fisher Scientific) in 12 microliters of Opti-MEM to each well or 200 ng of mRNA in 5 microliters of Opti-MEM and 400 ng of MessengerMax (Thermo Fisher Scientific) in 5 microliters of Opti-MEM to each well. Luciferase activity was recorded one day after transfection by adding 25 microliters of Bright-Glo (Promega) and

measuring activity using GloMax equipment (Promega). ZsGreen signal was recorded by acquiring cells using Flow activated cell cytometry (FACS) (Canto, BD Biosciences) and analyzing the results using FlowJo.

3. Results and Discussion

3.1 Aptamer 17 enhances *cis* mRNA expression

Miyakawa *et al.* reported the sequences of eight aptamers that could bind to eIF4G (Miyakawa et al., 2006). Five of these molecules (aptamers 2, 14, 17, 18, and 19) did not inhibit translation. We tested the shortest four of these five molecules (aptamers 2, 14, 17, and 19), which were 41 bases or less in length; aptamer 18, which is 70 residues long, was not tested. Oligonucleotides with a T7 promoter sequence followed by the aptamer sequence and a 20-base sequence that recognizes the wild-type luciferase sequence at its start codon (see supplementary Figure 1 for the primer sequences) were used to generate DNA templates for *in vitro* transcription. Uncapped, enzymatically capped and ARCA-capped mRNAs were produced using the aforementioned PCR matrices. mRNAs were transfected into human tumor cells (HEK cells) using Lipofectamine 2000, and luciferase activity was recorded. As presented in Figure 1A, uncapped mRNAs, even those with aptamer sequences at their 5' end, were not translated. However, detectable luciferase activity was observed for mRNAs that were enzymatically (Figure 1B) or co-transcriptionally (Figure 1C) capped. Remarkably, mRNAs with the aptamer 17 sequence consistently exhibited several fold increases in

luciferase activity compared with mRNAs without a 5' aptamer sequence and mRNAs with the aptamer 2 sequence (aptamers 14 and 19 moderately enhanced translation in certain experiments). The same results were obtained when mRNAs were transfected into mouse tumor cells (CT26 cells, Figure 1D) or when transfection reagents other than Lipofectamine 2000 (such as MessengerMax, which is a liposome optimized for mRNA transfection, Figure 1E) were used. The addition of the aptamer 17 sequence to different mRNAs, such as codon-optimized luciferase (Figure 1F) or ZsGreen (Figure 1G) with globin stabilization untranslated 5' and 3' sequences, enhanced the expression of reporter proteins. Thus, the aptamer 17 sequence is an optimization element that can function alone or in combination with other mRNA-optimizing sequences, such as stabilizing UTRs.

For gene therapy approaches, the mRNA must be deficient in triggering RNA sensors such as Toll-like receptors. This objective can be achieved by substituting modified nucleotides for canonical nucleotides, most notably by replacing canonical uridine with 1-methyl-pseudouridine. As shown in Figure 2, the aptamer 17 sequence could increase luciferase expression produced by mRNA molecules containing 1-methyl-pseudouridine instead of uridine, regardless of whether mRNAs are transfected into tumor cells (HEK, cells, Figure 2A) or untransformed human cells (immature dendritic cells, Figure 2B, or mature dendritic cells, Figure 2C).

In conclusion, the addition of an eIF4G-binding aptamer to the 5' UTR of ivt mRNA can be used as a universal method to increase the quantity of protein produced from recombinant mRNA. The numerous ongoing pre-clinical and clinical studies that involve evaluating the efficacy of mRNA-based therapies should take advantage of the method

reported here to increase the functionality of ivt mRNA and thereby achieve a higher therapeutic index with the same dose of mRNA or the same therapeutic index with a lower dose of ivt mRNA.

Acknowledgments

This work was supported by the University Research Priority Project (URPP) “Translational Cancer Research”.

Authorship Contributions

MT performed experiments and participate to the redaction of the manuscript

TK supported the study and participated to the redaction of the manuscript

SP designed the study, performed experiments and wrote the manuscript

Conflict of Interest Disclosures

Steve Pascolo is inventor of a patent on the use of eIF4F-directed aptamers to increase translation of mRNA (EP17198422)

Figure 1

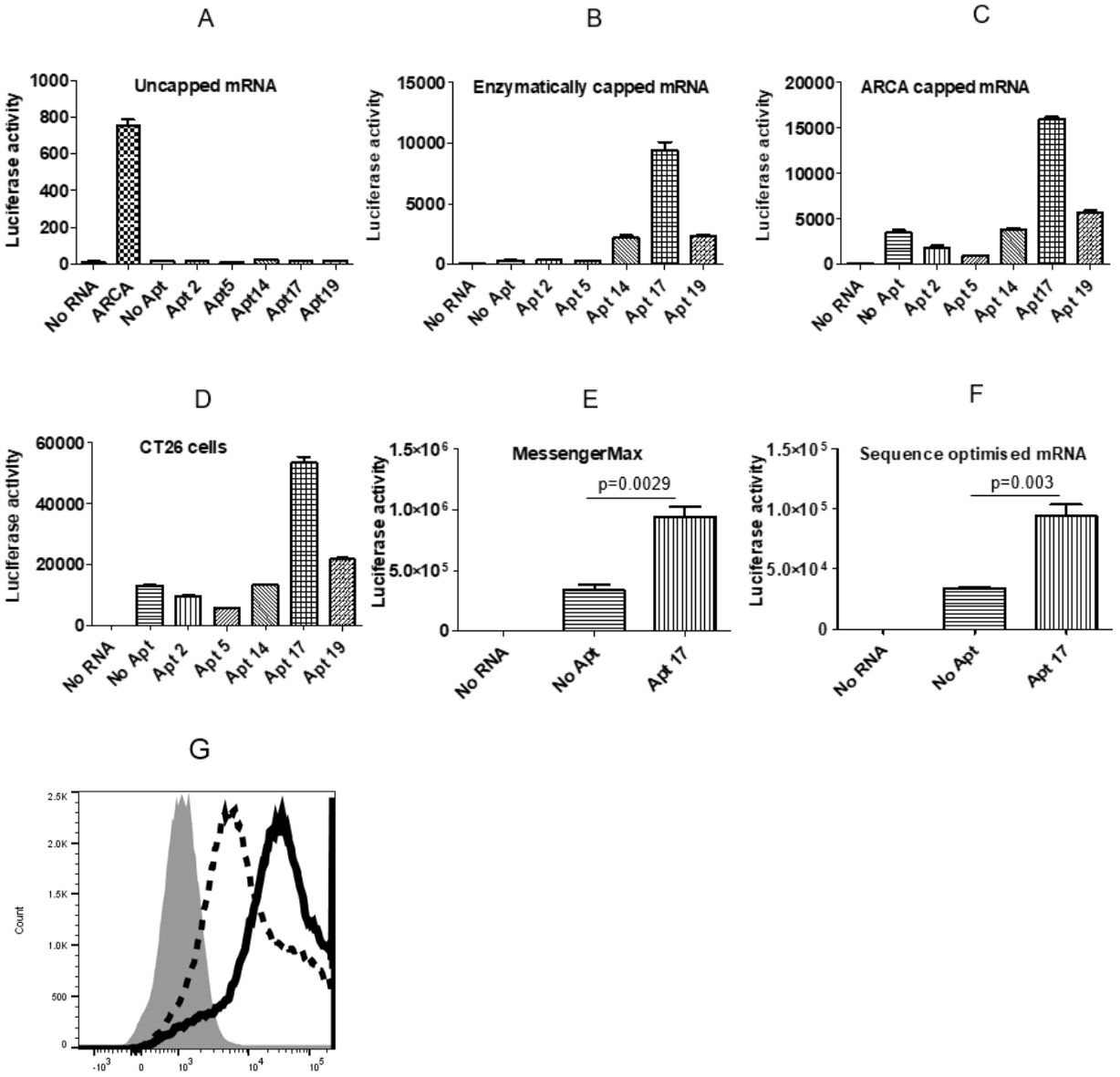

Figure 2

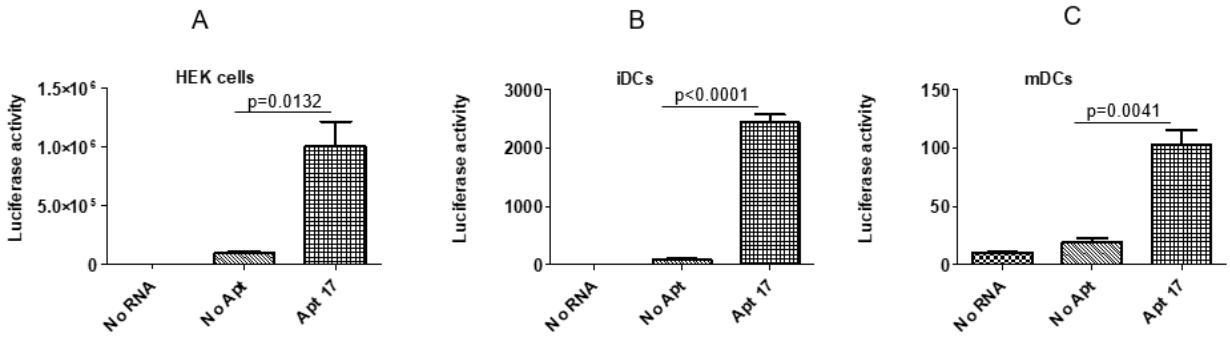

Figure legends

Figure 1: Effects of eIF4G-binding aptamers on the efficacies of ACGU ivt mRNAs. Uncapped (A), enzymatically capped (B) or ARCA-capped (C) mRNAs with (Apt 2, Apt 14, Apt 17 and Apt 19) or without (No Apt) a 5' eIF4G-binding aptamer sequence were transfected into HEK cells (A, B, C, E, F, G) or CT26 cells (D) using Lipofectamine 2000 (A, B, C, D, F) or MessengerMax (E, G). Twenty-four hours after transfection, luciferase activity was recorded (A to F, means and deviations for experiments performed in triplicate are shown). At that time, fluorescence was measured using FACS (G), with representative results presented: the filled histogram represents untransfected cells, the gray line represents cells transfected with an mRNA without an aptamer in its 5' UTR, and the black line represents cells transfected with an mRNA with the aptamer 17 sequence in its 5' UTR.

Figure 2: Effects of eIF4G-binding aptamers on the efficacies of ACGψ ivt mRNAs. Enzymatically capped mRNAs in which uridine residues were replaced by 1-methyl pseudouridine residues and the 5' UTR contained (Apt 17) or lacked (No Apt) the eIF4G-binding aptamer 17 sequence were transfected into HEK cells (A), immature human dendritic cells (B) or mature human dendritic cells (C) using MessengerMax. Twenty-four hours after transfection, luciferase activity was recorded. The means and deviations for experiments performed in triplicate are shown.

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Deoxycoformycin exerts anti-tumour activity by demasking endogenous RNA and triggering Toll-like Receptor 3

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Keywords: deoxycoformycin, pentostatin, RNA-methylation, Toll-like Receptor-3

Abstract

Mammalian RNA, which is heavily modified, does not trigger innate immune receptors when delivered in liposomal particles *in vitro* (Kariko et al., 2005). In contrast, using delivery by protamine nanoparticles (Tusup and Pascolo, 2017), we found that mammalian RNA induces TNF-alpha in human peripheral blood mononuclear cells (PBMCs). However, these mammalian RNA particles did not induce type I interferon in PBMCs. RNA from lower organisms induced TNF-alpha as well as type I interferon in PBMCs when formulated with protamine. Thus, RNA modifications in higher organisms, which are primarily inosines and methylations prevent the recognition of this nucleic acid by receptors associated with triggering type I interferon. We hypothesize that these modifications are potentially druggable. Direct or indirect inhibition of adenosine deaminase activity reduces inosine production and has also been reported to lower the methylation index. Hence, we screened inhibitors of adenosine deaminases and identified pentostatin (2-deoxycoformycin) as a drug that enhances the capacity of endogenous RNAs to trigger type I interferon. Used for over thirty years as an anti-cancer drug (particularly against hairy cell leukaemia), pentostatin, which is not directly toxic to tumour cells, has an unknown mode of action. Here, we show that pentostatin is an immunomodulating drug that promotes endogenous RNA sensing as a danger signal for TLR3 and induces type I interferon, which triggers effective anti-cancer responses.

Foreign or mislocalized endogenous RNAs are recognized as danger signals by several cellular immune receptors from the Toll-like receptor (TLR) and RIG-I-like receptor (RLR) families. The TLR family members TLR7, TLR8 and TLR3 located on endosomal membranes (reviewed in (Kawai and Akira, 2010)) as well as cytosolic retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated protein 5 (MDA5) receptors in the RLR family (Hornung et al., 2006; Kato et al., 2006) promptly react to such RNA stimuli. Several modifications of RNA bases or backbones inhibit nucleic acid recognition by these dedicated immune receptors, thereby labelling metazoan cellular RNA as self and preventing immune stimulation by endogenous RNAs. Indeed, mammalian RNA delivered in liposomes does not trigger cytokine production in TLR-transfected cells or antigen-presenting cells *in vitro* (Kariko et al., 2005). In contrast, we found that total RNA extracted from mammalian cells or tissues and formulated in 120 nm protamine nanoparticles (Figure 1A and Extended Figure 1A) induced TNF-alpha production in human peripheral mononuclear cells (hPBMCs) (Figure 1B). Other inflammatory cytokines, such as IL-6, IL-17, and interferon-λ, were also induced by all tested RNAs in the protamine formulation (Supplementary Table 1). By comparing the immunostimulation profiles of natural RNAs packaged in protamine or liposome particles, we confirmed that only the protamine-RNA formulation unmasked the potential of human RNAs to induce TNF-alpha (Figures 1B and C). Meanwhile, independent of packaging type, alpha interferon is only produced by hPBMCs when the RNA originates from more basal organisms (*Escherichia coli* (*E. coli*), *Saccharomyces cerevisiae* (*S. cerevisiae*), and *Drosophila melanogaster* (*D. melanogaster*)) but not humans (Figure 1D and E). Cytokines such as IL-12 and IL-1alpha are also preferentially induced by RNAs from more basal organisms (Supplementary Table 1). The protamine-RNA nanoparticle triggers endosomal TLR7, TLR8 and TLR3 when both unmodified ssRNAs and dsRNAs are condensed (Extended Figure 1B). Because TLR7 or TLR3 stimulation triggers type I interferon production and TLR8 stimulation promotes inflammatory cytokine production, we concluded that mammalian RNA modifications block recognition by TLR3 and/or TLR7. We postulated that the pathways leading to these immunosuppressive RNA modifications might be druggable.

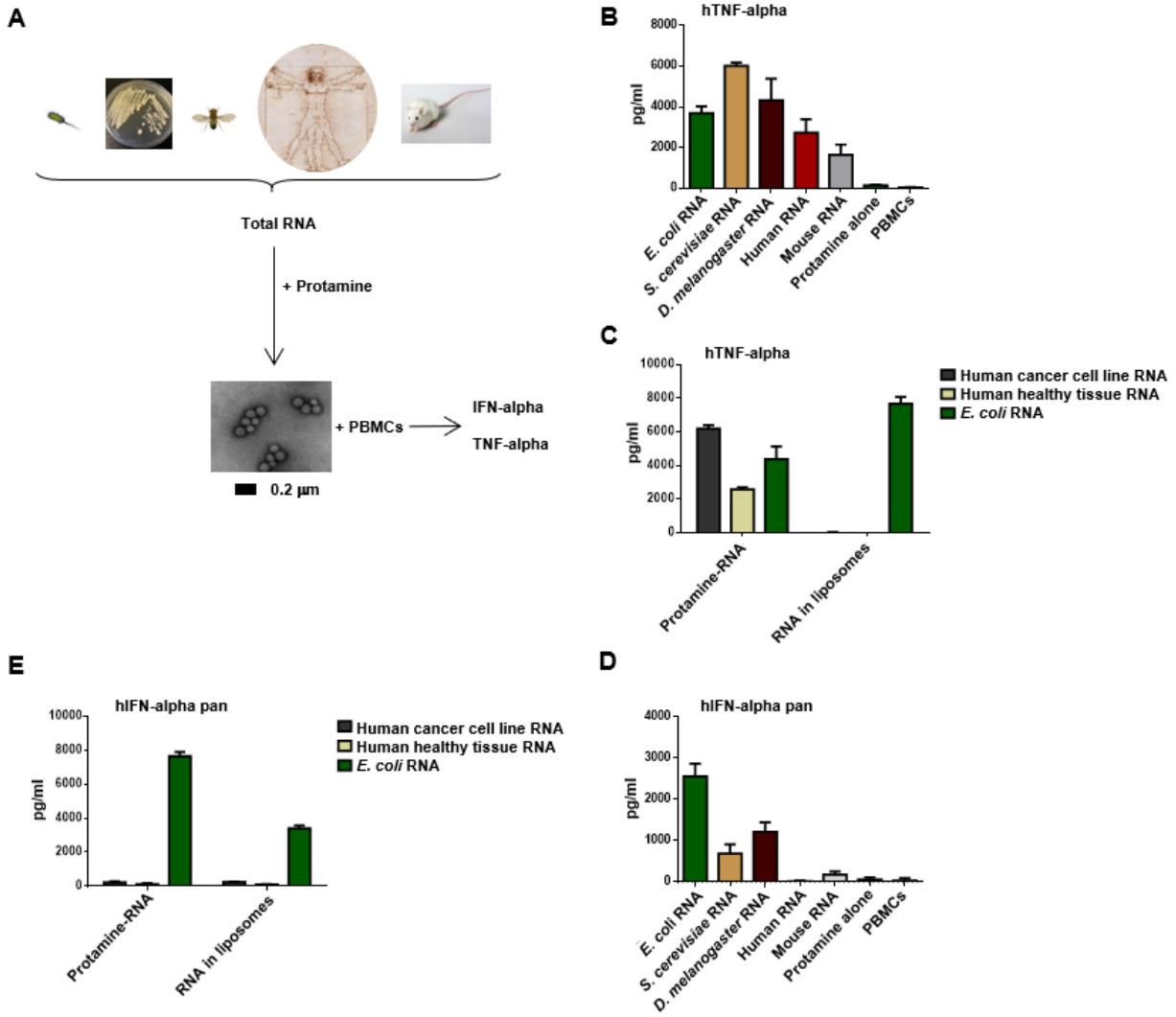
Inosine and base/sugar methylation are abundant, naturally occurring modifications to mammalian RNA (Wang and He, 2014) that block RNA recognition by immune sensors (Jung et al., 2015; Sarvestani et al., 2014). To unmask the type I interferon-inducing potential of endogenous RNA, we screened drugs that altered the pathways responsible for introducing these modifications. Inhibition of adenosine deaminases impedes inosine production and simultaneously induces adenosine accumulation. This hinders S-adenosyl-L-homocysteine (SAH) hydrolase activity, which leads to an imbalance in production of the methylation donor S-adenosylmethionine (SAM) and results in decreased SAM/SAH methylation index (Glazer and Hartman, 1980; Johnston and Kredich, 1979; Kredich, 1980). By testing direct (erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA) (North et al., 1983) and deoxycoformycin (Agarwal et al., 1977)) and indirect (metformin (Ouyang et al., 2011) and methotrexate (Riksen et al., 2006)) adenosine deaminase inhibitors, we found that deoxycoformycin (pentostatin, Nipent), the most specific and effective adenosine deaminase inhibitor, increased the capacity of total RNA extracted from *in vitro*-treated tumour cells to trigger type I interferon in hPBMCs (Figure 2A and Supplementary Figure 2A). The TNF-alpha induction capacity of packaged RNA was not significantly affected (Figure 2B). Similarly, RNA extracted from organs of mice injected intravenous with pentostatin induced more alpha interferon production in hPBMCs than RNA extracted from organs from untreated mice (Figure 2C and D and Extended Figure 2B and 2C). Pentostatin (2'-deoxycoformycin), a natural adenosine analogue produced by the bacterium *Streptomyces antibioticus* and the fungus *Emericella nidulans*, has been reported to be the most potent adenosine deaminase inhibitor (Agarwal et al., 1977). T cells are particularly dependent on adenosine deaminase activity. Deoxycoformycin has been tested to treat diverse types of leukaemia and lymphoma and is reported to be efficacious against hairy cell leukaemia (Spiers et al., 1984), chronic lymphocytic leukaemia (Dillman et al., 1988), prolymphocytic leukaemia (Dohner et al., 1993), Hodgkin lymphoma (Braiteh et al., 2006), and adult T-cell lymphoma, especially cutaneous T cell lymphoma (Grever et al., 1983; Kurzrock et al., 1999). However, pentostatin is not directly toxic to tumour cells ((Wilson et al., 1998) and Extended Figure 2D), and its mode of action is indirect and still unknown 35 years after its introduction into clinical practice.

Our *in vitro* and *ex vivo* data prompted us to investigate whether the anti-cancer effects of pentostatin are mediated by the immune system. Indeed, we found that the drug only exhibited anti-cancer activity in tumour models in which immune checkpoint inhibitors (reviewed in (Grosso and Jure-Kunkel, 2013) were also efficacious (Extended Figure 3A). In the two models, we found that immune mediators were required to promote anti-tumour responses after Pentostatin intravenous injection: type I interferon receptor (Figure 3A) and T cells (Figure 3B and Extended Figure 3B). Pentostatin did not significantly enhance the frequency of circulating anti-cancer T-cells (extended Figure 3C). Mice cured by pentostatin were protected from tumour challenge (Figure 3B). Histological studies indicated that after pentostatin injection, tumours were infiltrated with T cells (Figure 3C). Heterozygous reporter mice carrying luciferase reporter genes under the control of an interferon-beta promoter (Lienenklaus et al., 2009) were used to monitor IFN-beta induction in tumour-bearing mice. After pentostatin injection, a luciferase signal was detected in the tumour, further demonstrating the indirect (only in tumor, Figure 3D and not seen *in vitro* on mouse splenocytes incubated with Pentostatin, Extended Figure 3D) immunostimulating role of pentostatin. The local induction of type I interferon in tumours and the requirement of type I interferon receptor for pentostatin activity are particularly striking: before 1984, when pentostatin was introduced, hairy cell leukaemia was treated with recombinant alpha interferon. To further investigate the link between RNA-sensing and pentostatin, we tested the efficacy of the drug in TLR3, TLR7 and mitochondrial antiviral-signalling protein (MAVS, deficient signal transduction from RIGI and MDA-5) knock-out (KO) mice. The efficacy of pentostatin was decreased only in the TLR3 KO (Figure 4A and Extended Figure 4A). Accordingly, TRIF KO mice also did not respond to the drug treatment (Extended Figure 4B). MAVS deletion had no effect on pentostatin efficacy. In addition, pentostatin induced only mild differential gene expression in the kidneys (only a slight increase in cellular respiration as can be anticipated from the reported increase in mitochondrial biomass in pentostatin-treated cells (Garcia-Gil et al., 2016), Figure 4C), and no induction of endogenous retroviruses (Extended Figure 4C). This is excluding the possibility of chromatin reorganization and immunostimulation by endogenous retroviruses as previously reported for DNA methylation inhibitors (Chiappinelli et al., 2015). Our experiments demonstrated that pentostatin acts *in vivo* by

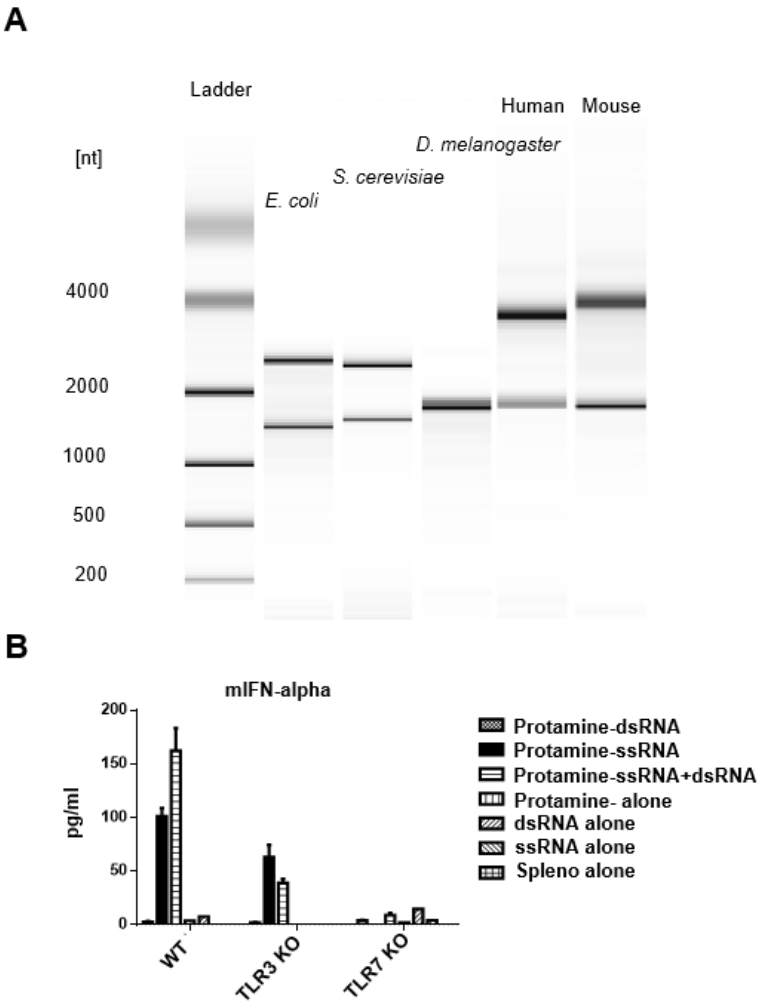
sensing endogenous RNA through the immune system and triggering an immune response. The following two types of RNA modifications block recognition by TLR3: (1) the creation of inosine in double stranded RNA by adenosine deaminase acting on RNA and (2) the methylation of the base or sugar. RNA-seq analysis of the total RNA extracted from organs from pentostatin-treated mice showed similar occurrence of inosine in mRNA from untreated and treated mice (Figure 4B). This finding agrees with previous reports indicating that pentostatin does not inhibit adenosine deaminases acting on RNA (Kim et al., 1994). Meanwhile, previous reports show that cells from pentostatin-treated patients showed an impaired ability to methylate nucleic acids (DNA and RNA) *ex vivo*. This day-lasting inability to methylate RNA post-pentostatin treatment was demonstrated for ribose 2′-O-methylation, which blocks RNA recognition by TLR3 and TLR7 in human cells (De Clercq et al., 1972; Jung et al., 2015). Based on these findings, we concluded that pentostatin promotes TLR3 by decreasing 2′-O-methylation of endogenous RNA. Because intravenous pentostatin injection induced a systemic decrease in the methylation index but type I interferon induction only in tumours *in vivo*, we postulated that demodified dsRNA arises from dying cells in the tumour microenvironment.

Our experiments demonstrate that silencing endogenous RNA immune sensing by inhibiting methylation is a druggable process. The anti-cancer drug pentostatin, which is not directly toxic to tumour cells and has an unknown mode of action when used to treat cancer patients, unmasks endogenous RNA and thereby promotes TLR3-TRIF signalling to induce the production of type I interferon in tumours. Thus, pentostatin, which is classified as a chemotherapy drug, should be reclassified as an immunotherapy drug. Its rational utilization with chemotherapies and immune checkpoint inhibitors should be evaluated in view of the actual mode of action revealed here. We anticipate that the original mode of pentostatin action will allow repurposing of this drug for combination treatments incorporating the induction of type I interferon at tumour sites into standard-of-care treatments.

Figure 1


A
Total RNA
+ Protamine
+ PBMCs
IFN-alpha
TNF-alpha
0.2 µm
B
hTNF-alpha
pg/ml
E. coli RNA
S. cerevisiae RNA
D. melanogaster RNA
Human RNA
Mouse RNA
Protamine alone
PBMCs
C
hTNF-alpha
pg/ml
Human cancer cell line RNA
Human healthy tissue RNA
E. coli RNA
Protamine-RNA
RNA in liposomes
E
hIFN-alpha pan
pg/ml
Human cancer cell line RNA
Human healthy tissue RNA
E. coli RNA
Protamine-RNA
RNA in liposomes
D
hIFN-alpha pan
pg/ml
E. coli RNA
S. cerevisiae RNA
D. melanogaster RNA
Human RNA
Mouse RNA
Protamine alone
PBMCs

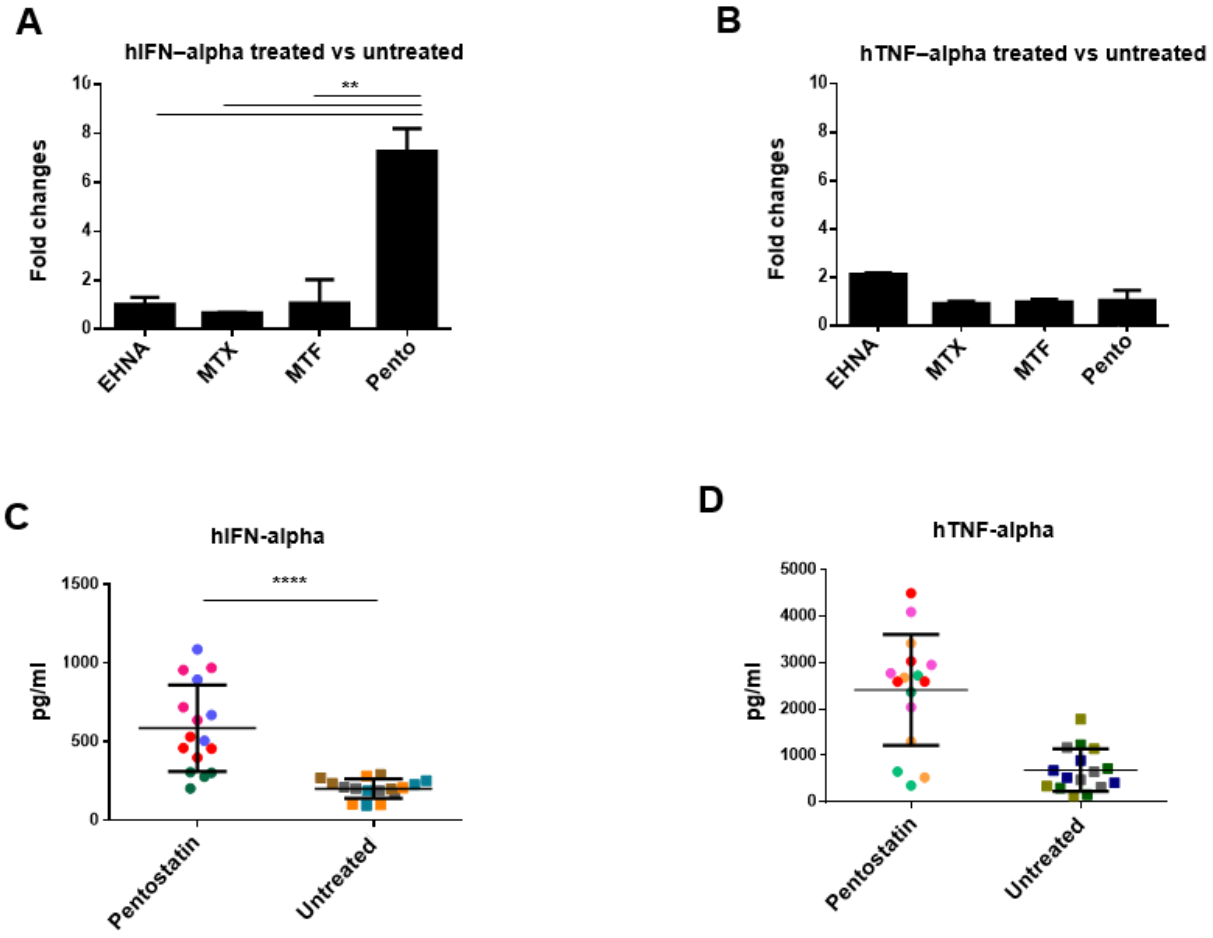
Extended figure 1



Supplementary table 1

Multiplex cytokine ELISA		IFN-α 2	IFN-β	IFN-λ	IFN-γ	IL-1α	IL-4	IL-5	IL-6	IL-8	IL-10	IL-12p70	IL-13	IL-15	IL-17	IL-23	TNF-α
Protamine-RNA	Bacteria (*E. coli*)	5550.8 (+/- 198.3)	ND	6929 (+/- 59.1)	OR	1581.42 (+/- 142.6)	ND	1309 (+/- 124.2)	27212.9(+/- 8787.9)	16375.4 (+/- 840.74)	584.2 (+/- 507.9)	5336.6(+/-361.2)	264.7 (+/- 98.3)	28.4 (+/- 13.3)	833.4 (+/- 491.5)	ND	14625.1 (+/- 2036.6)
	Yeast (*S. cerevisiae*)	589.4 (+/- 472.4)	ND	5152 (+/- 289.4)	OR	1231.3 (+/- 1.5)	ND	1164 (+/- 16.5)	24717.9(+/- 1175.8)	20144.7 (+/- 1202.6)	412.9 (+/- 506.3)	5311.8 (+/-10.7)	176.2 (+/- 39.8)	3.4 (+/- 12)	449.7 (+/- 87.1)	ND	27983.5 (+/- 2671.9)
	Insect (*D. melanogaster*)	1883.3 (+/- 408.3)	ND	6065.3 (+/- 62.5)	108493.2 (+/- 7935.8)	1415.5 (+/- 61.9)	ND	1341.9 (+/- 48.7)	26401.1 (+/- 260.5)	11938 (+/- 416.7)	715.1 (+/- 618.5)	2702.6 (+/- 518.6)	213.5 (+/- 84.5)	ND	619.8 (+/- 127.9)	ND	18865.4 (+/- 1214.5)
	Mammal (Human)	35.1 (+/- 84.7)	ND	4459.5 (+/- 45.2)	79848.7 (+/- 5027.5)	746 (+/- 19.3)	ND	1129 (+/- 65.3)	20580.7 (+/- 724.7)	14019.5 (+/- 1491.9)	1039.2 (+/- 1277.6)	628.4(+/- 10.8)	214.4 (+/- 8.2)	ND	303.6 (+/- 64.7)	ND	12309.1 (+/- 931.2)
	Mammal (Murine)	89.1 (+/- 9.3)	ND	4796.7 (+/- 597.8)	109409.9 (+/- 2355.6)	326.6 (+/-96)	ND	ND	17981.2 (+/- 1317.5)	9476.6 (+/- 1176.5)	809.3 (+/- 5.9)	629.9 (+/- 268.4)	339.6 (+/- 76.6)	4.3 (+/- 3.4)	564.1 (+/- 464.8)	ND	12105.9 (+/-511.6)
	Protamine alone	ND	ND	ND	ND	570.2 (+/- 41.6)	ND	ND	ND	5879.1 (+/- 405.93)	ND	ND	ND	ND	ND	ND	ND	ND
	PBMCs alone	77.7 (+/- 94.3)	ND	ND	ND	458.4 (+/- 73.3)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
RNA alone	Bacteria (*E. coli*)	ND	ND	ND	ND	ND	ND	ND	18191 (+/- 55.3)	OR	6798.7 (+/- 169.8)	ND	ND	ND	ND	ND	4009.6 (+/- 37)
	Yeast (*S. cerevisiae*)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	609 (+/- 37.4)	ND	ND
	Insect (*D. melanogaster*)	ND	ND	ND	ND	ND	ND	ND	ND	1880.9 (+/- 2771.8)	ND	ND	ND	ND	ND	ND	ND
	Mammal (Human)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	860 (+/- 314.8)	ND	ND
	Mammal (Murine)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	1040.5 (+/- 109.2)	ND	ND

Figure 2


A
hIFN–alpha treated vs untreated
Fold changes
**
EHNA
MTX
MTF
Pento
B
hTNF–alpha treated vs untreated
Fold changes
EHNA
MTX
MTF
Pento
C
hIFN-alpha
pg/ml
****
Pentostatin
Untreated
D
hTNF-alpha
pg/ml
Pentostatin
Untreated

Extended figure 2

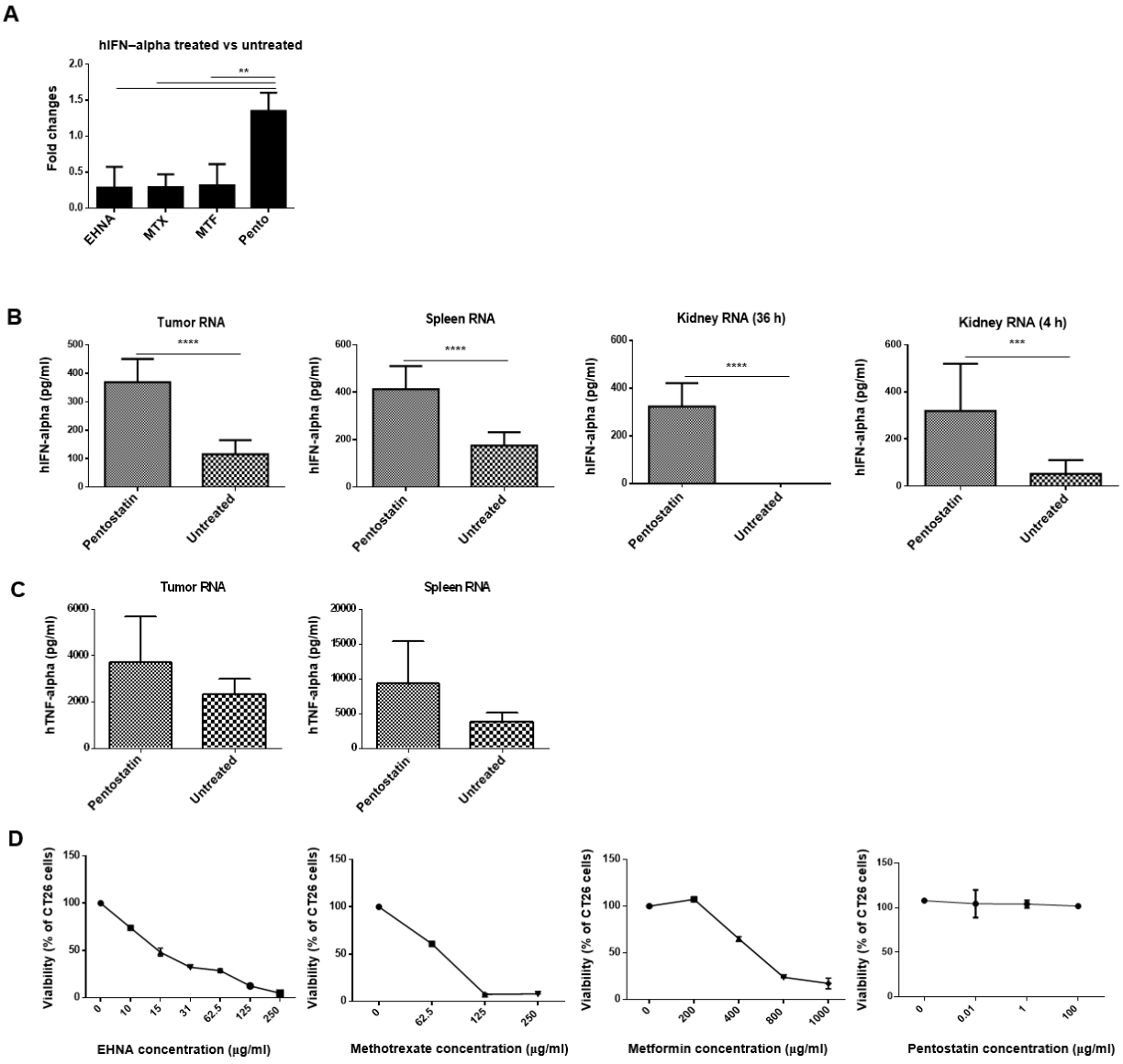
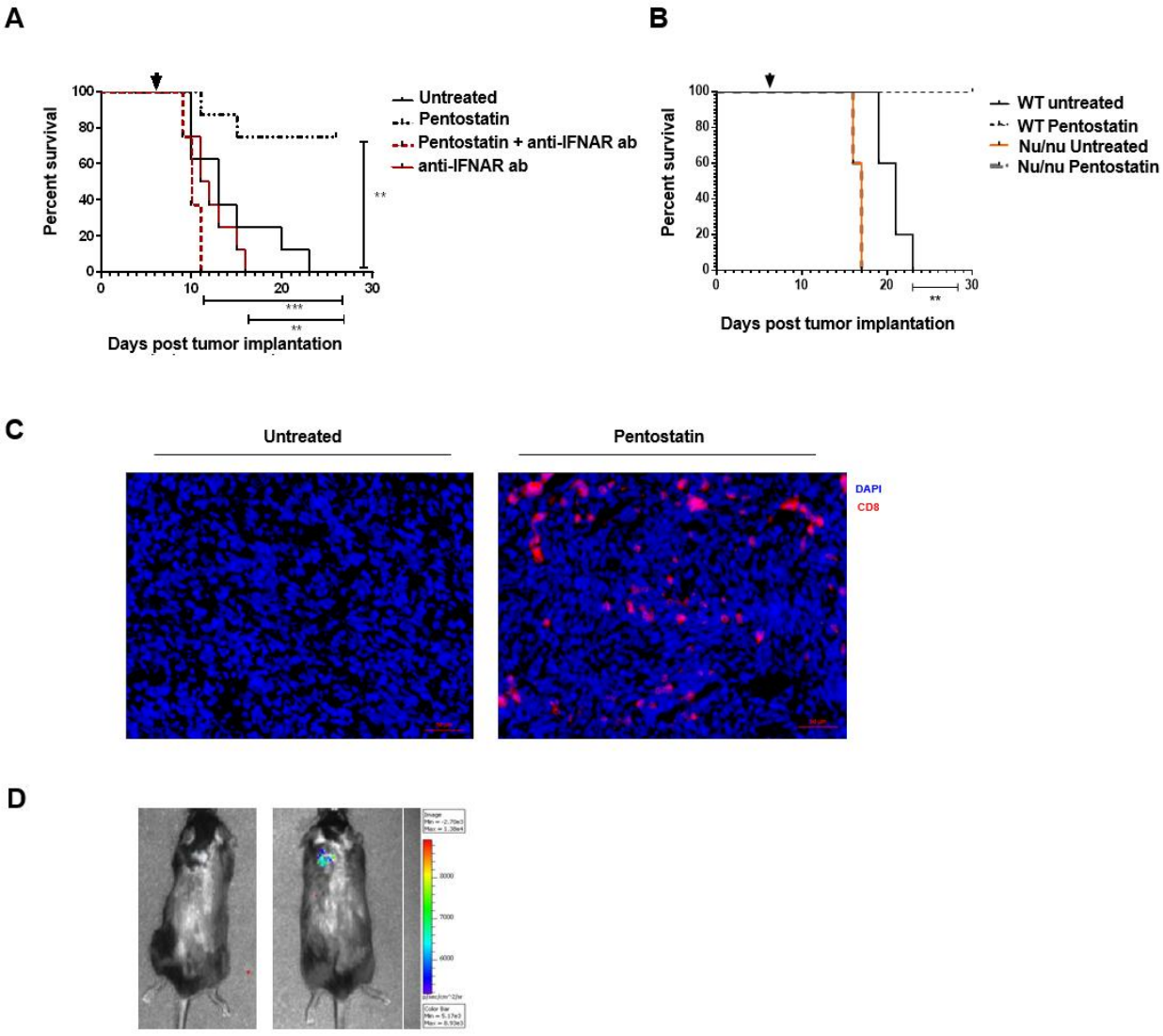


Figure 3


A
Percent survival
Days post tumor implantation
Untreated
Pentostatin
Pentostatin + anti-IFNAR ab
anti-IFNAR ab
B
Percent survival
Days post tumor implantation
WT untreated
WT Pentostatin
Nu/nu Untreated
Nu/nu Pentostatin
C
Untreated
Pentostatin
DAPI
CD8
D

Extended figure 3

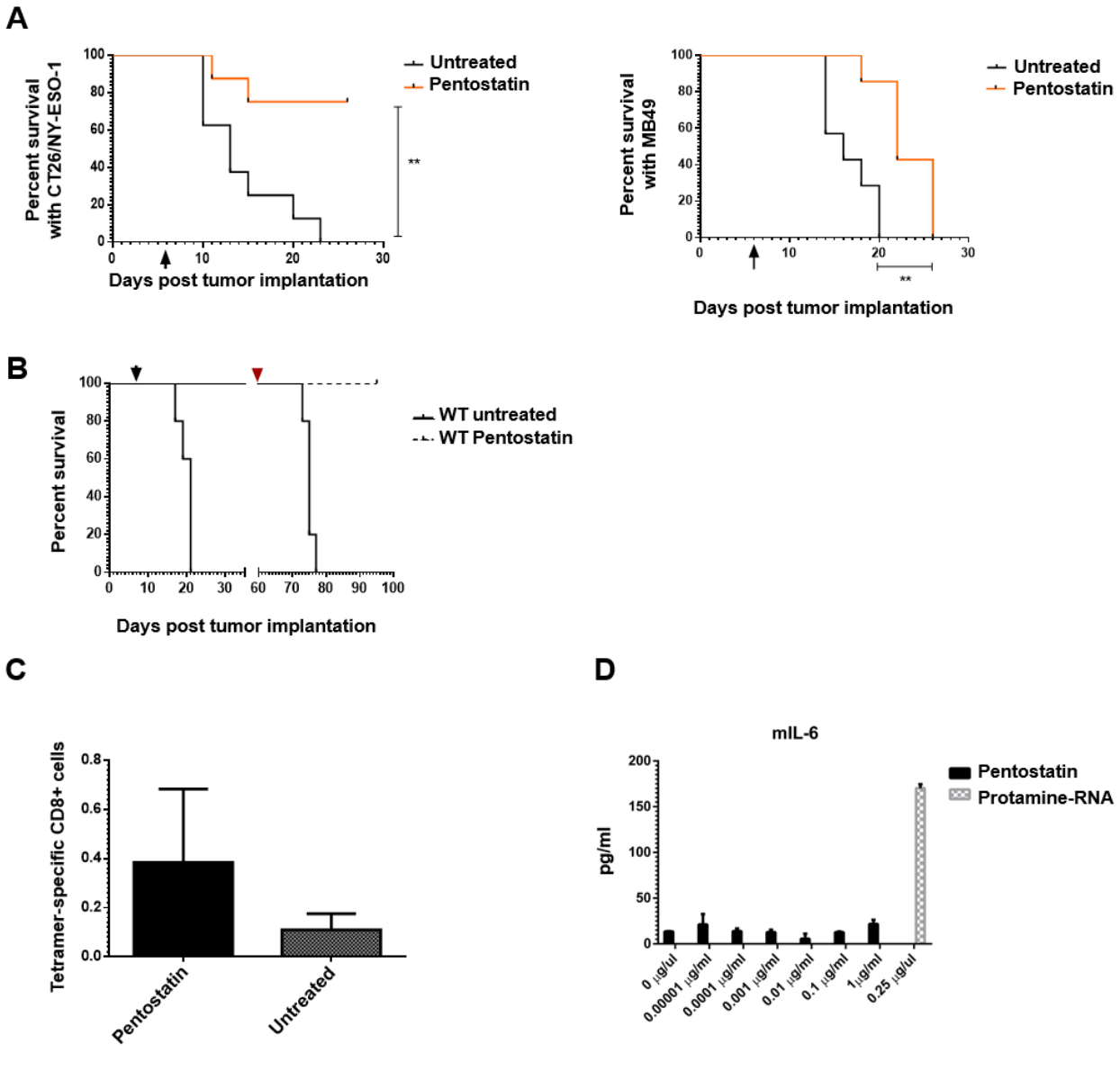
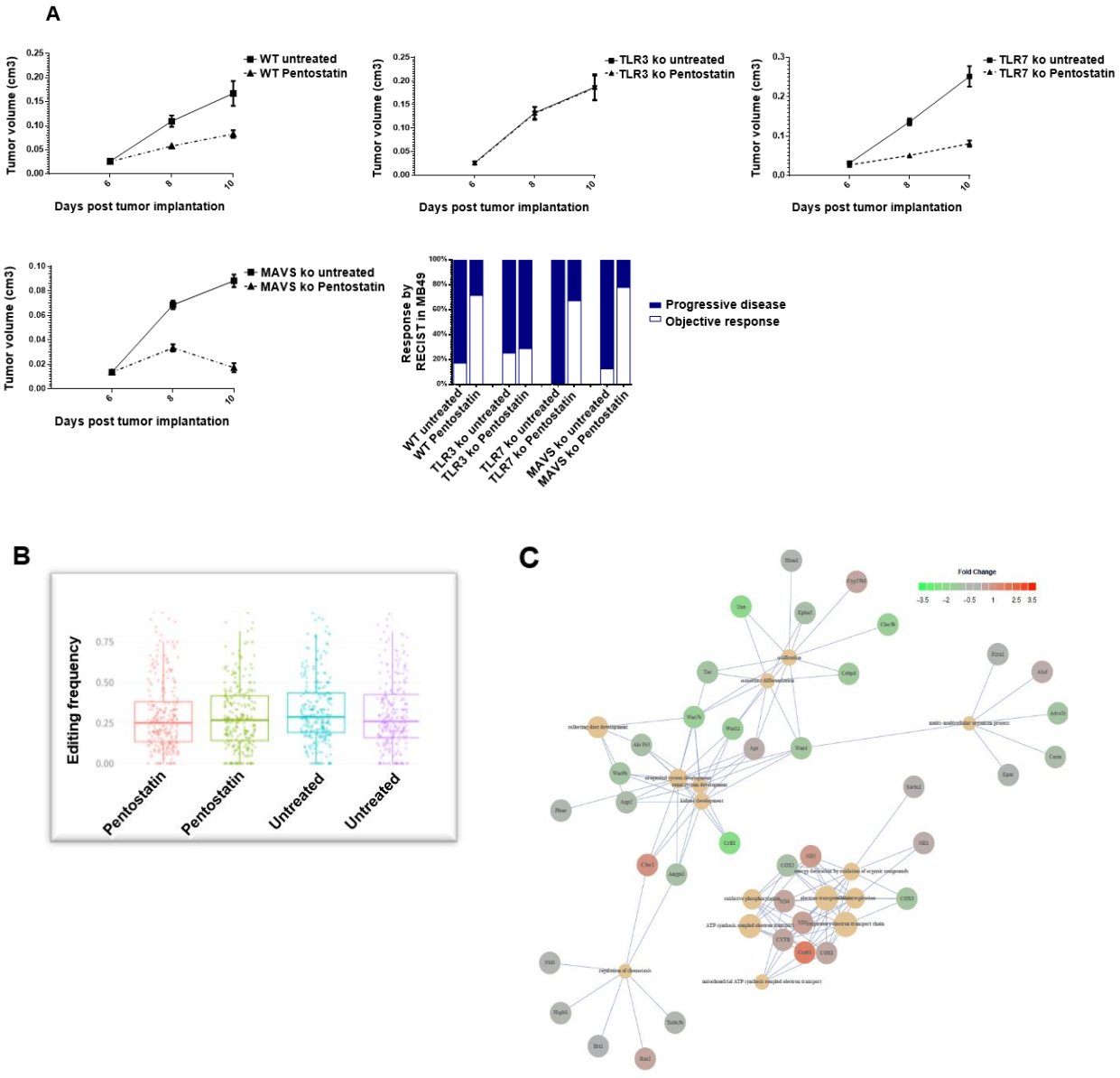
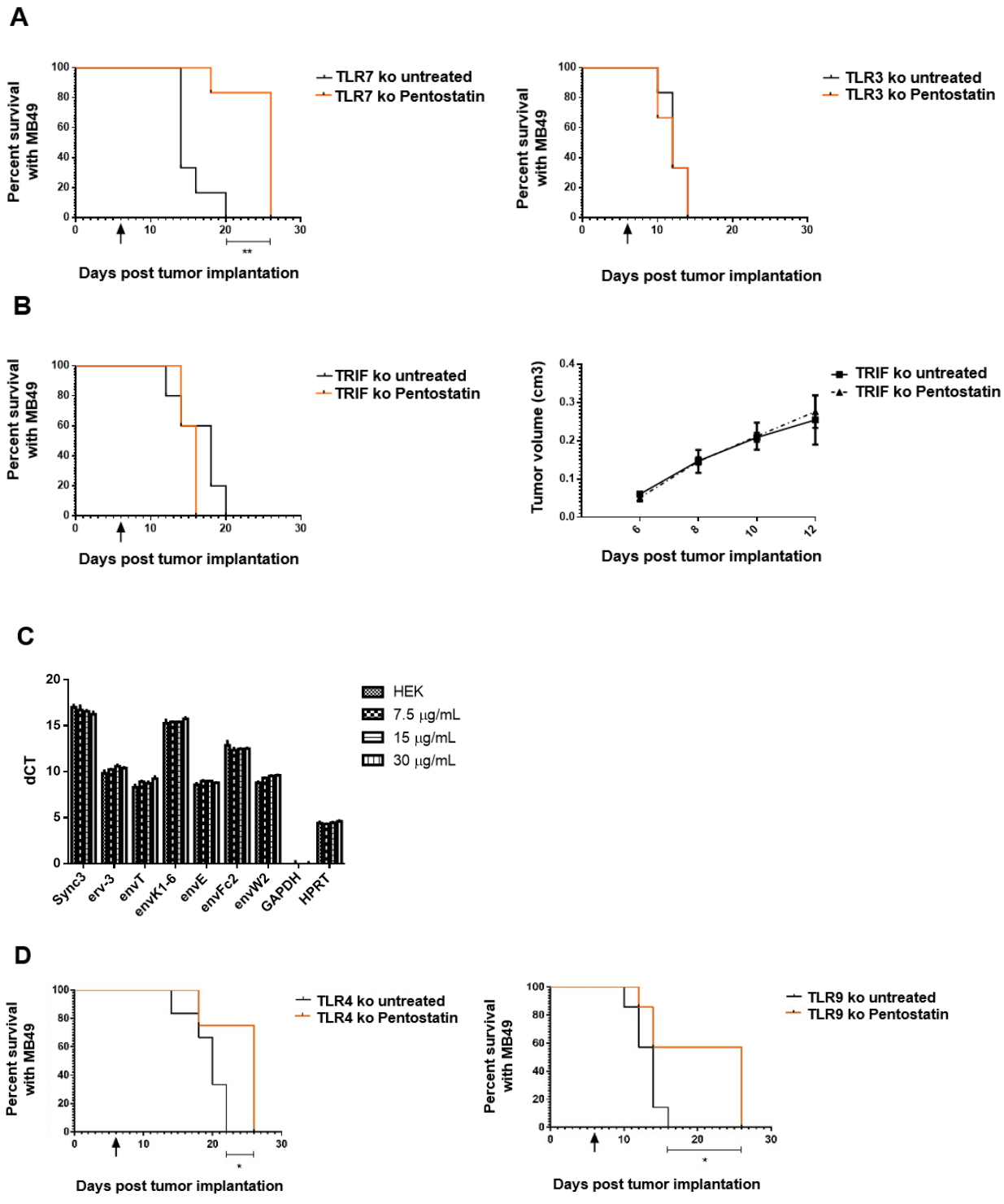


Figure 4


A
Tumor volume (cm3)
Days post tumor implantation
WT untreated
WT Pentostatin
TLR3 ko untreated
TLR3 ko Pentostatin
TLR7 ko untreated
TLR7 ko Pentostatin
MAVS ko untreated
MAVS ko Pentostatin
Response by RECIST in MB49
Progressive disease
Objective response
WT untreated
WT Pentostatin
TLR3 ko untreated
TLR3 ko Pentostatin
TLR7 ko untreated
TLR7 ko Pentostatin
MAVS ko untreated
MAVS ko Pentostatin
B
Editing frequency
Pentostatin
Pentostatin
Untreated
Untreated
C
Fold Change

Extended figure 4



Legends

Figure 1: Total mammalian RNA in protamine-RNA nanoparticles induces hTNF-alpha but not hIFN-alpha in hPBMCs.

A) *In vitro* study workflow. Total RNA (1 µg) extracted from *Escherichia coli* (*E. coli*), *Saccharomyces cerevisiae* (*S. cerevisiae*), *Drosophila melanogaster* (*D. melanogaster*), humans and mice were combined with protamine (2 µg) to generate 120 nm nanoparticles and incubated with hPBMCs. After overnight incubation, IFN alpha and TNF alpha were measured by ELISA in the corresponding supernatants.

B) Human TNF-alpha (hTNF-alpha) was induced by all natural RNAs when packaged in protamine-RNA. hTNF-alpha quantified in supernatants from hPBMCs incubated with protamine-RNA formulations (*E. coli*, *S. cerevisiae*, *D.* melanogaster, human, mouse) with protamine and PBMC only controls (n=3).

C) Protamine-RNA nanoparticles containing total RNA from human cells revealed the potential of these RNAs to induce hTNF compared to RNA in liposomes. hTNF-alpha in supernatants from PBMCs incubated with protamine-RNA (human cancer cell line, human healthy tissue and *E. coli* RNA) and the same RNA in liposomes (n=3).

D) Human IFN-alpha (hIFN-alpha) is induced only by RNA from lower organisms (*E. coli*, *S. cerevisiae* and *D. melanogaster*). hIFN-alpha quantified in the supernatants of hPBMCs incubated with protamine-RNA (*E. coli*, *S. cerevisiae, D. melanogaster* and human) and showing protamine and PBMC only controls (n=3).

E) Protamine-RNA nanoparticles exhibit the same hIFN-alpha induction profile as RNA in liposomes. hIFN-alpha from PBMC cell supernatants incubated with protamine-RNA formulations (human cancer cell line, human healthy tissue and *E. coli* RNA) and the same RNA in liposomes (n=3). Error bars are the mean±s.d.

Extended Figure 1:

A) Agilent 2100 Bioanalyzer measurement of total RNA (eukaryote total RNA Nano chip) lanes: Ladder, *E. coli*, *S. cerevisiae*, *D. melanogaster*, human RNA, mouse RNA (from left to right; 500 ng).

B) Mouse IFN-alpha quantification in supernatants from splenocytes incubated with protamine and double-stranded RNA (dsRNA), protamine and single-stranded (ssRNA), protamine and ssRNA+dsRNA, protamine alone, dsRNA alone, ssRNA alone and medium only.

Supplementary Table 1

Human Cytokine Multiplex ELISA quantification in supernatants from hPBMCs incubated overnight with protamine-RNA nanoparticles (2 µg:1 µg) or protamine alone, peripheral blood mononuclear cells (PBMCs) alone and each total RNA alone as controls. Total RNA from a bacterium (*E. coli*), yeast (*S. cerevisiae*), insect (*D. melanogaster*), and mammal

(human and murine) for human IFN Alpha 2 (IFN-α 2), IFN Beta (IFN-β), IFN Lambda (IFN-λ 1, 2 and 3), and IFN Gamma (IFN-γ); human Interleukin-1 Alpha (IL-1α), Interleukin-4 (IL-4), Interleukin-5 (IL-5), Interleukin-6 (IL-6), Interleukin-8 (IL-8), Interleukin-10 (IL-10), Interleukin-12 (IL-12p70), Interleukin-13 (IL-13), Interleukin-15 (IL-15), Interleukin-17 (IL-17) and Interleukin-23 (IL-23); and Human Tumour Necrosis Factor-Alpha (TNF-α). Abbreviations: ND represents not detected, and OR represents outside of the value range. Values represent the mean±s.d of respective duplicates in pg/ml.

Figure 2: Pentostatin enhanced the potential of total RNA from cells or organs to induce hIFN-alpha in hPBMCs incubated with protamine-RNA nanoparticles.

A) and B) Total RNA extracted from CT26 cells treated with the half maximal inhibitory concentration (IC50) of EHNA (erythro-9-(2-hydroxy-3-nonyl) adenine), MTX (methotrexate), MTF (metformin), and 0.3 µg/ml (near clinical dose) Pento (Pentostatin) for 72 h formulated packaged with protamine and incubated overnight in hPBMCs. Ratios are calculated according to the following formula: (protamine-RNA from treatment/protamine-RNA untreated)/(RNA alone from treatment/RNA alone untreated). A significant fold change was observed for hIFN alpha induction after pentostatin treatment. All the values represent three independent experiments with ELISA measurements for (A) hIFN-alpha pan and (B) hTNF-alpha. Error bars are the mean±s.d. ** based on the T-test, p=0.0054 Pento vs. MTF, p=0.0024 Pento vs. MTX, and p=0.003 Pento vs. EHNA. C) and D) Total RNA extracted from mouse kidneys 18 h after mice were i.v. injected with 10 µg pentostatin or were not injected combined with protamine to generate protamine-RNA nanoparticles, which were incubated with hPBMCs overnight. (C) hIFN- alpha pan and (D) hTNF-alpha were quantified in supernatants. Biological duplicates with experimental duplicates and technical duplicates of each value were performed Error bars are the mean±s.d. **** based on T-test, p<0.0001 is extremely significant.

Extended Figure 2:

A) Total RNA was extracted from HEK293 cells treated with the IC50 of EHNA (erythro-9-(2-hydroxy-3-nonyl) adenine), MTX (methotrexate), MTF (metformin), and 0.3 µg/ml of pentostatin for 72 h and then packaged with protamine to generate nanoparticles, which were incubated overnight with hPBMCs. The ratios were calculated according to the following formula: (protamine-RNA from treatment/protamine-RNA untreated)/(RNA alone from treatment/RNA alone untreated). A significant fold change was observed for hIFN alpha induction after pentostatin treatment. All the values represent three independent experiments with ELISA measurements for hIFN-alpha. Error bars are the mean±s.d. ** based on the T-test, p=0.0065 Pento vs. MTF, p=0.0019 Pento vs. MTX, and p=0.0055 Pento vs. EHNA. B) and C) Total RNA extracted from mouse tumors (CT26) and spleen after 18 h and from kidneys 4 and 36 h after the mice were i.v. injected with 10 µg of pentostatin or were untreated mice and packaged with protamine to generate protamine-RNA nanoparticles, which were incubated with hPBMCs overnight.

(B) hIFN-alpha and (C) hTNF-alpha were quantified in the supernatants. Pentostatin treatment significantly enhanced the potential of endogenous RNA to induce hIFN-alpha in all three compartments. Bars correspond to biological replicates and experimental replicates of each biological replicate. Error bars are the mean±s.d.

D) Cytotoxicity quantification with LDH assay. CT26 cells were incubated with different doses of EHNA, MTX, MTF and pentostatin. The viability during three days was calculated according to the following formula: mean (A490 Treatment)-A490 blank)/mean (A490 Untreated)-A490 blank)*100. Pentostatin showed no toxicity at any tested dose in CT26 cells. Error bars are the mean±s.d. **** based on the T-test. $p<0.0001$ is extremely significant.

Figure 3: Pentostatin activity *in vivo* in tumour-bearing mice depends on type I interferon and T cells.

A) Survival of CT26/NY-ESO-1 tumour-bearing mice treated with pentostatin, pentostatin+anti-IFNAR ab, anti-IFNAR ab and untreated. Pentostatin is effective for treating CT26/NY-ESO-1-established tumours. Its activity is prevented by the anti-IFNAR ab; n=8 in each group. Black arrows point to the day of pentostatin treatment. The significant differences between the survival curves were calculated with the log-rank (Mantel-Cox) test. *** p=0.0007 for pentostatin vs. pentostatin+anti-IFNARab; ** p=0.0025 for pentostatin vs. untreated, and ** p=0.0014 for pentostatin vs. anti-IFNARab.

B) Survival of CT26 tumour-bearing nude (nu\nu) and wild-type (WT) mice. T cell-deficient nude mice showed no response to pentostatin treatment; n=5 in each group. Black arrows point to the day of pentostatin treatment. The significant differences between the survival curves were calculated with the log-rank (Mantel-Cox) test. ** p=0.0019.

C) Immunofluorescent staining of CT26 tumour cryo-sections from mice 36 h after pentostatin treatment or from untreated mice. CD8+ cells visible only after pentostatin treatment. DAPI staining is for nucleated cells, and PE-Texas Red staining marks CD8+ cells; 20x magnification.

D) Bioluminescence measurements *in vivo* in MB49 tumour-bearing heterozygous reporter IFN-$\beta^{+/\Delta\beta\text{-luc}}$ mice injected with pentostatin on day 6 or not treated. Pictures on the right; the tumour-specific signal only present in the pentostatin-injected mice 18 hours after injection.

Extended Figure 3:

A) Survival of CT26/NY-ESO-1 tumour-bearing mice (n=8 in each group) and MB49 (n=7 in each group) mice treated with pentostatin and untreated mice. Pentostatin cures 67% of CT26/ NY-ESO-1 mice and provides tumour growth stability in the MB49 tumour model. Black arrows point to the day of pentostatin treatment. The significant differences between survival curves were calculated with the log-rank (Mantel-Cox) test. ** p=0.0025, left graph and p=0.0012, right graph.

B) CT26/NY-ESO-1 tumour-bearing mice treated with pentostatin showed immune memory in a tumour re-challenge experiment. Pentostatin-cured animals were re-challenged with same tumours 60 days after the first tumour was implanted and showed no tumour growth for over 90 days. In parallel, an untreated group was implanted with tumours and showed the expected progression (n=5). Black arrows point to the day of pentostatin treatment, and red arrows represent the day of tumour re-challenge.

C) Percent of tetramer-specific CD8+ cells measured in the blood from mice 4 days after pentostatin treatment (n=8) or from untreated mice (n=5). The percentage of CD8+ cells specific for the NY-ESO-1 epitope increased after pentostatin-treatment.

D) Mouse IL-6 quantification in supernatants from splenocytes incubated with different doses of Pentostatin, or 0.25 mg/ml Protamine-RNA (unmodified luciferase encoding mRNA) nanoparticles. Error bars are the mean±s.d.

Figure 4: Pentostatin activity *in vivo* in tumour-bearing mice requires TLR3.

A) MB49 tumour volume measurements in tumour-bearing mice (WT, TLR3 KO, TLR7 KO and MAVS KO, left to right) with or without pentostatin treatment. Pentostatin controlled tumour growth in all mice except TLR3 KO mice. Error bars are the mean±s.e.m. Summarized responsiveness (WT (n=7), TLR3 KO (n=7), TLR7 KO (n=6) and MAVS KO (n=7)) in stacked bars according to whether it was a progressive disease, with an at least 20% increase in tumour volume, an objective response, with stable disease (tumour size increased less than 20% or decreased up to 30%) or a partial response (decrease in tumour volume of at least 30%). Objective responses for day 10 vs. day 6 shown for pentostatin-treated WT, TLR7 KO and MAVS KO mice; the TLR3 KO mice showed no objective response.

B) Inosine quantification in kidney RNA according to the editing frequency in RNA-seq datasets from pentostatin-treated (18 hours post-injection) and untreated mice. The distributions of editing frequencies in each sample at sites listed in RADAR were visualized as boxplots. Y-axis shows editing frequency, corresponding to a fraction of aligned reads with edited base as listed in RADAR. Each point in the figure is a genomic site listed in RADAR and having a depth of coverage of at least five reads. Editing frequency and thereby inosine incorporation were not affected in the total RNA of pentostatin-treated mice.

C) Enriched gene ontology (GO) terms were visualized as a network of enriched GO terms and differentially expressed genes connected to them. Genes with lower and higher expression in treated samples were coloured green and red, respectively.

Extended Figure 4

A) Survival curves for TLR7 KO and TLR3 KO and MB49 tumour-bearing mice treated with pentostatin or untreated (n=7). Pentostatin promoted longer survival times in TLR7 KO mice, whereas TLR3 KO mice showed no responses to pentostatin treatment. The significant differences between the survival curves were calculated with the log-rank (Mantel-Cox) test. ** p=0.0015;

B) Survival curves and tumour growth in TRIF KO MB49 tumour-bearing mice treated with pentostatin and untreated (n=5). TRIF KO mice did not respond to pentostatin treatment. Error bars are the mean±s.e.m. Black arrow points to the day of pentostatin treatment.

C) Induction of endogenous retroviruses quantified by real-time PCR in total RNA extracted from HEK cells incubated 2 days with 0, 7.5; 15; 30 µg/ml of Pentostatin. All values represent duplicates and dCT value is calculated by substracting the average value obtained for GAPDH to the experimental value. Error bars are the mean±s.e.m.

D) Survival curves in TLR4 ko and TLR9 ko MB49 tumor-bearing mice treated with Pentostatin and untreated (n=7). Both TLR4 ko and TLR9 ko responded to Pentostatin treatment. Error bars are the mean±s.e.m. Black arrow points to the day of pentostatin treatment.

Methods and materials

Mice

WT and nude mice were purchased from Envigo. Tlr3-/-, Tlr7-/-, Tlr4-/-, Tlr9-/-, MAVS-/- and TRIF-/- (all on have C57BL/6 genetic background) were bred in house. IFN beta/Luc reporter mice were kindly provided by Dr. Stefan Lienenklaus (University of Hannover) and bred in house.

Age-matched (6–12 weeks) female and male animals were used throughout experiments. Animal experiments were approved by the regulatory authorities (license 175/2015 "Study of the immunological anti-cancer effects of Pentostatin, an ADA inhibitor"). All mice were kept in accordance with regulations from the Laboratory Animal Services Center (LASC) at the University Hospital of Zurich.

Tumour cell lines

CT26/NY-ESO-1 is a murine colorectal cancer cell line expressing NY-ESO-1 and was kindly provided by Professor Hiroyoshi Nishikawa (Muraoka et al., 2010). MB49 bladder cancer cell line was kindly provided by Dr. Sonia Domingos-Pereira (University Hospital of Lausanne, Switzerland). All cell lines were kept in RPMI medium containing 10% fetal bovine serum (Thermo Fisher Scientific) and 1% 200 mM L-glutamine and implanted subcutaneously (1 million in 200 microliters of PBS). Cells were tested for pathogens and approved from LASC regulatory office. In house testing for mycoplasma was performed during the time of experiments.

E. coli, *S. cerevisiae* and *D. melanogaster*, human and mouse RNA

E. coli, human muscle, human pancreas, mouse embryo and HeLa RNAs were ordered from Ambion (Thermo Fisher Scientific). Tablets of dormant *S. cerevisiae* were reactivated in water solution containing 1% D-glucose and 0.1% yeast extract kept at 28-30 degrees with slow steering for 6-8 h. *D. melanogaster* flies were provided by Julia Falschlunger (Institute of Molecular Life Sciences, University of Zurich).

RNA preparations

Total RNA from cells or tissues was obtained using miRNeasy Mini Kit (Qiagen) according to manufacturer instructions. For the isolation of RNA from yeast, a combination of lyticase/zymolase mixtures prior to RNA isolation was employed in combination with RLT

(Qiagen) buffer. All RNAs were checked either on 1.2% agarose containing 1% MOPS-EDTA and 0.75% of 37 % formaldehyde (Sigma) or on Agilent RNA 600 Nano chips using 2100 Agilent bioanalyzer. Agilent bioanalyzer and software were kindly provided from Silvia Lang (Gastroenterology Department, University Hospital Zurich).

Protamine RNA formulations

Protamine (IPEX) was purchased from Meda and stored at 4 degrees. Total RNA diluted in water at 0.5 ug/ul was combined with Protamine diluted in water at 0.5 ug/ul. Particle size was checked using light scattering spectroscopy (Zeta Sizer, Malvern). Stability and further properties of Protamine-RNA formulations have been published (Rettig et al., 2010; Tusup and Pascolo, 2017). 1 ug of diluted total RNA mixed with 2 ug of diluted Protamine are incubated at room temperature for 10 minutes before addition of 200 microliters of complete medium containing one million of human PBMCs and incubation 24 hours at 37°C.

ELISA measurements

Human IFN-alpha pan (Mabtech) and TNF alpha (Biolegend) were measured in fresh and frozen cell culture supernatants. Values were obtained on Biotek reader at 450 nm using Gen5 software. VeriPlex human cytokine 16-Plex ELISA (PBL) was employed for multiple cytokine measurements. Image J was used as a software for quantifying values obtained from chemiluminescent measurement of VeriPlex ELISA.

Drugs

Pentostatin (Sigma) stored as powder at -20, was diluted in PBS for a working concentration of 10 mg/ml and used immediately. EHNA (Sigma), Metformin (Merck Serano) and Methotreaxate (Pfizer) were stored and diluted according to manufacturer instructions.

Tumor models and treatment

Mice were inoculated subcutaneously with $1x10^6$ tumor cells. Tumor sizes were measured unblinded with a caliper every second day for calculating tumor volumes using the equation $(a^2 \times b)/2$ (a, width; b, length). Animals were euthanized when exhibiting signs of impaired health or when tumor volume exceeded 1 cm^3. Pentostatin (Sigma) was injected intravenously when tumors were palpable (day 6 for MB49 and day 7 for CT26). When indicated, 1mg of anti-IFNAR1

blocking antibody (MAR1-5A3, BioXCell) solution was injected intraperitoneally at the same day than Pentostatin.

Bioluminescent in vivo imaging

Bioluminescence in vivo imaging of tumor bearing IFN beta reporter mice was performed on IVIS Lumina (PerkinElmer) at specific time point post Pentostatin injection. Immediately prior to measurements D-Luciferin (Synchem) dissolved in PBS (15 mg/ml stock) and sterile filtered was injected (150 µg/g intraperitoneally per mouse). Emitted photons from live animals were quantified 10-20 minutes post Luciferin injections with an exposure time of 1 min. Regions of interest (ROI) were quantified for average radiance (photons s−1 cm−2 sr−1) (IVIS Living Image 4.0).

Immunofluorescence staining

CD8+ staining was performed on 6-8-µm sections of cryo-preserved tumors. Cryosections were fixed for 5 min in -20 cold methanol and equilibrated for 5 min in PBS. After three washes in PBS containing 0.1% Tween 20 (PBST), slides were blocked for 1h at RT in 12% BSA in PBS containing 0.1% NP-40 (blocking buffer). CD8 antibody (eBioscience, Thermo Fischer Scientific) PE conjugated at 1:1000 dilution in blocking buffer were incubated overnight at 4 degrees. Sections were washed three times in PBST. Nuclei were stained with DAPI (Sigma) (1:2000). After three washes with PBST, sections were quickly washed in water, mounted in fluorescent mounting-medium and air-dried for 2 h at room temperature. Immunofluorescence images were acquired using Zen Pro Axio Zeiss microscope and software.

Flow cytometry

Monoclonal antibodies for extracellular staining included CD4, CD8 and CD11c (eBioscience, Thermo Fischer Scientific). Quantification of NY-ESO-1-specific CD8+ T cells with APC-labelled H-2Dd tetramer loaded with the 81-88 NY-ESO-1 peptide (TCmetrix) was made using mouse blood five days after Pentostatin injection. Collected blood was washed in fluorescence-activated cell sorting (FACS) buffer and tetramer staining was performed in FACS buffer simultaneously with CD4 and CD8 stainings. Erythrocyte lysis with BD FACS lysing solution was performed after 1h of incubation with antibodies. After lysis samples were washed in PBS

and suspended in FACS buffer for measurement on FACS Canto I with FACS Diva BD software. For quantification and analysis of cell populations of interest FlowJo X was used.

LDH assay

Different cell lines were seeded (5000 cells per well) in 300 ul of complete medium in 48 well plate. One day later, supernatants are replaced by 200 microliters of complete medium. Different doses of tested drug were added in 200 ul. After three days of growth wells were washed gently with MEM (no serum) and frozen in 500 microliters of PBS at -80°C. Next day, plates were placed at room temperature 3 hours at least, with one hour of shaking, then plates were spun for 5 minutes at 1500 rpm. From plates 10 microliter supernatants were transferred in 96 well plate with addition of 40 µL substrate for LDH from Cytotox96 kit (Promega). 10 microliters of PBS plus 40 µL of substrate was used for background value. Reactions were stopped with the addition of 40 µL stop solution and plates were measured at OD 490nm. The percentage of cells was calculated according to the formula: (experimental value-background)/(untreated cells-background)x100.

Reverse transcription and quantitaive Polymerase chain reaction (qPCR)

Total RNA extracted by miRNeasy kit (Qiagen). 1 µg of RNA was reverse transcribed using the High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems) as recommended by the manufacturer. An equivalent of 16 ng of initial RNA was used per well in qPCR (Roche) reactions using primers specific for house keeping genes (Glyceraldehyde-3-phosphate dehydrogenase - GAPDH and hypoxanthine–guanine phosphoribosyltransferase - HPRT) as well as primers specific for several endogenous retroviruses (from (Chiappinelli et al., 2015)). The dCT value was calculated by substracting the average value obtained for GAPDH to the experimental value.

Statistical analyses and data presentation

All results are expressed as mean±s.d. Biological replicates were used in all experiments unless stated otherwise. Unpaired two-tailed Student's t-test was used for comparison of two groups. One-way analysis of variance (ANOVA) was performed when more than two groups were compared, and when determined significant ($P<0.05$).

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Chapter 9

Generation of Immunostimulating 130 nm Protamine–RNA nanoparticles

Marina Tusup and Steve Pascolo

Abstract

Nanoparticles of defined size can be easily obtained by simply mixing Protamine, a pharmaceutical drug that is used to neutralize heparin after surgery, and RNA in the form of oligonucleotides or messenger RNA. Depending on the concentrations of the two reagents and their salt contents, homogenous nanoparticles with a mean diameter of 50 to more than 1000 nm can spontaneously be generated. RNA is a danger signal because it is an agonist of for example TLR-3, -7, and -8; therefore, Protamine–RNA nanoparticles are immunostimulating. We and others have shown in vitro that nanoparticle size and interferon-alpha production by human peripheral blood mononuclear cells (PBMCs) are inversely correlated. Conversely, nanoparticle size and TNF-alpha production by PBMCs are positively correlated (Rettig et al., Blood 115:4533–4541, 2010). Particles of less than 450 nm are most frequently used for research and clinical applications because they are very stable, remain polydispersed and induce interferon-alpha proteins, which are a natural antiviral and anticancer protein family with 12 members in humans. Herein, we describe a method to generate 130 nm nanoparticles as well as some of their physical and biological characteristics.

Key words RNA, Protamine, Nanoparticles, Toll like receptor, Interferon-alpha, TNF-alpha

1 Introduction

Danger signals are immunostimulatory molecules that can stimulate surface, intravesicular, and intra-cytosolic receptors. Danger signals can be used as vaccine adjuvants as well as monotherapies to induce/boost innate and adaptive immunity against pathogens and tumor cells [1]. Toll-like receptors (TLRs) are specialized in the recognition of danger signals [2] and are expressed in different immune cells and different subcellular locations. When triggered, TLRs induce specific intracellular activation pathways that can result in the expression of different types of innate immune response molecules, such as alpha interferons (all 12 human alpha interferon

Electronic supplementary material: The online version of this chapter (doi:10.1007/978-1-4939-6481-9_9) contains supplementary material, which is available to authorized users.

Thomas Kramps and Knut Elbers (eds.), *RNA Vaccines: Methods and Protocols*, Methods in Molecular Biology, vol. 1499,
DOI 10.1007/978-1-4939-6481-9_9,

protein family members) and/or TNF-alpha. Unmodified single-stranded RNA (ssRNA) is recognized by human TLR-7 (expressed, for example, in plasmacytoid dendritic cells, the main producers of interferon-alpha) and human TLR-8 (expressed, for example, in monocytes, which are capable of producing large amounts of TNF-alpha) [3]. Thereby, ssRNA can trigger a broad range of immune cells and activation pathways [4, 5]. To protect it from RNase activity and successfully deliver it to endosomes in which TLR-7 and TLR-8 are located, ssRNA must be formulated for example in lipoplexes or polyplexes (or lipopolyplexes). Several teams including us have developed RNA polyplex formulations based on Protamine, a natural cationic peptide that is used as a drug to inhibit heparin and that also spontaneously associates with nucleic acids [4–9]. Thus, immunostimulating polyplexes can be produced for injections into humans, and we used these polyplexes as vaccines [10] or peptide vaccine adjuvants [11, 12] as experimental immunotherapy in cancer patients. Remarkably, the size of the Protamine–RNA polyplexes can be specifically and simply determined by regulating the salt concentration in the Protamine and RNA components before mixing to promote the spontaneous formation of specifically sized particles [13]. As the salt concentration decreases, the particle size decreases. In this manner, particles from 50 nm to above 1000 nm can be easily produced. Surprisingly, the size of the particles dictates their immunostimulating features. Only particles less than 450 nm efficiently stimulate plasmacytoid dendritic cells and thereby induced high levels of interferon-alpha. Larger particles activate monocytes but not plasmacytoid dendritic cells and thereby trigger the production of TNF-alpha but not alpha interferons [13]. Thus, Protamine–RNA particles of different sizes are ideal, versatile tools to activate immune cells of interest, such as in dendritic cell-based vaccination [14]. Meanwhile, because alpha-interferons are of great interest for anticancer and antivirus therapies, we have focused our research on Protamine–RNA particles that are less than 450 nm in size. These particles are further being developed as immunomodulatory drugs (Tusup and Pascolo, ongoing studies) and are also used as adjuvants for mRNA vaccines [15]. This article presents detailed methods for generating immunostimulating Protamine–RNA particles approximately 130 nm in size.

2 Materials

For dilutions, resuspension, and analysis, we use ultrapure water (prepared by purifying deionized water to attain a sensitivity of 18.2 M Ω cm at 25 °C, 4 ppb TO—Milli-Q® Advantage A10 ultrapure water).

2.1 Particle Components

1. Protamine. Pharmaceutical Protamine is available at two concentrations: 1000 (1 ml neutralizes 1000 IU of heparin) or 5000 (1 ml neutralizes 5000 IU of heparin). To generate particles of 130 nm, we use Protamine Ipex 5000 from MEDA Pharmaceuticals (Protamine hydrochloride 5000 IU/ml, *see* **Note 1**). The Protamine is stored at 4 °C (*see* **Note 2**).
2. Messenger RNA. Highly purified mRNA coding for firefly Luciferase was produced by in vitro transcription and provided by BioNTech Ag (Prof. Ugur Sahin, Mainz, Germany). The transcript is approximately 1800 bases and contains canonic A, C, G and U residues with a 5′ cap and 3′ poly-A tail (*see* **Note 3**). The mRNA is stored at −20 °C (*see* **Note 4**).

2.2 Particle Analyzer

1. Zetasizer (Malvern) 3000HSA Particle Analyzer equipped with PCS software.
2. Transparent cuvettes (Disposable cuvettes, 1.5 ml, semi-micro, Brand + CO Gmbh, Germany).

2.3 Stimulation of Human Blood Cells

1. Heparin tubes (BD vacutainer LH 17 IU/ml, cat no. 367526).
2. Ficoll solution (Ficoll-Paque™ Plus, GE Healthcare Life Sciences, 17-1440-02).
3. Pasteur pipets.
4. Centrifuge with controllable brake (Eppendorf™ Model 5810 Centrifuge).
5. Phosphate Buffered Saline (PBS) (without Ca and Mg, pH 7.2, sterile, not for infusion, Kantonsapotheke Zurich, Switzerland).
6. 15 ml Falcon tubes.
7. Complete medium: RPMI medium 1640 (Sigma cat no. R0883) containing 10% heat-inactivated fetal bovine serum (Gibco, Life Technologies, Thermo Fischer Scientific), 1:100 antibiotics (Penicillin-Streptomycin Thermo Fischer, cat no. 15140-122) and L-Glutamine (200 mM, Merck Milipore cat no. K 0282).
8. 96-well U bottom plate (Falcon).
9. Humidified 37 °C CO_2 incubator.

2.4 Detection of Alpha Interferons

1. Pan interferon-alpha kit (MABTECH, cat no. 3425-1A-6).
2. ELISA reader (Biotek, ELx808 Absorbance Reader, software Gen 5, 2.07 version).

3 Methods

Carry out all procedures at room temperature unless otherwise specified.

3.1 Production of the 130 nm Protamine–RNA Nanoparticles

This step is performed under laminar flow using sterile equipment.

1. Prepare a 0.5 mg/ml Protamine solution (*see* **Note 5**) by diluting 10 µl of Protamine 5000 in 280 µl of water.
2. Prepare a 0.5 mg/ml RNA solution (*see* **Note 5**) by diluting the RNA stock with the appropriate amount of water.
3. Add an equal amount of Protamine solution to the RNA solution (*see* **Note 6**), and pipet quickly up and down at least ten times (*see* **Note 7**).
4. Leave the solution at room temperature for 10 min (*see* **Note 8**).

3.2 Particle Size Measurement

1. Dilute 40 µl of the Protamine–RNA formulation (containing 10 µg of RNA and 10 µg of Protamine) with 1 ml of water (*see* **Note 9**).
2. Set the viscosity of the analyzer to 0.89 (select within the software setting either measurement in water or a viscosity of 0.89). As shown in Fig. 1, particles made in the conditions described above are approximately 130 nm in size with a polydispersity index (PDI) of less than 0.3 (*see* **Note 10**).

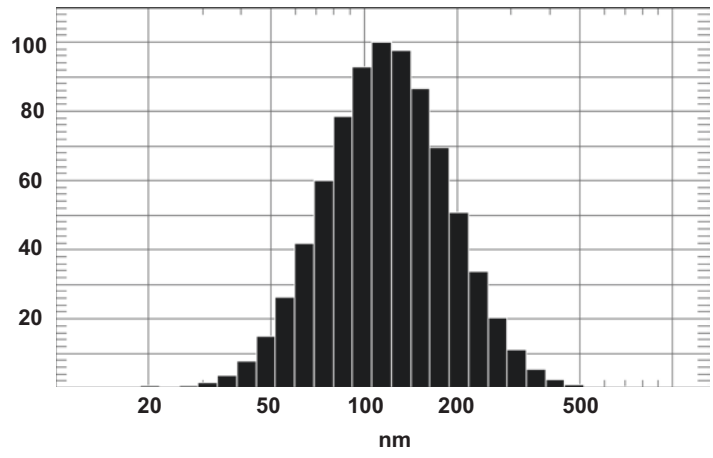

Fig. 1 Measurement of particle size displaying "Intensity-Weighted Gaussian Distribution Analysis" for solid particles. Average size is 131 nm and PDI is 0.214

3.3 Evaluation of Interferon-Alpha-Inducing Capacities in Human Blood Cells

This step is performed under laminar flow using sterile equipment.

1. Place 5 ml of fresh venous blood (collected from a healthy donor in a heparin tube, *see* **Note 11**) in a 15 ml Falcon tube.
2. Add a Pasteur pipet to the tube, and make sure that the tip of the Pasteur pipet is at the bottom of the tube.
3. Using a 5-ml pipet attached to a pipet aid, pipet 5 ml of Ficoll inside the Pasteur pipette. This fluid will underlay the blood. Elevate the Pasteur pipet slightly if the Ficoll does not flow easily into the bottom of the tube. When the maximum of Ficoll has gone in the 15 ml tube (when the level of Ficoll in the Pasteur pipet is at the level of the blood in the tube), close the Pasteur pipet with an index finger and lift it out of the Falcon tube. Discard the Pasteur pipet.
4. Carefully place the tube in a centrifuge with a balance tube on the opposite side of the rotor and centrifuge the tubes at 805 × *g*, 20 °C for 20 min without braking (*see* **Note 12**).
5. Prepare a 15 ml Falcon tube with 10 ml PBS.
6. Carefully place the tube containing blood and Ficoll under laminar flow, aspirate the upper phase (plasma) up to approximately 5 mm above the interface, and collect up to 3 ml of the interface liquid (it will contain some cells in plasma and some Ficoll) using a 5-ml pipet attached to a pipet aid. Perform small rotations with the pipette tip to collect cells that may be close to the tube wall.
7. Dilute the 3 ml collected in 10 ml PBS (tube prepared in **step 5**).
8. Mix by inverting the tube three times and place it in a centrifuge (equilibrated with a balance tube on the opposite of the rotor), and centrifuge the tubes at 453 × *g*, 20 °C for 10 min.
9. Discard the supernatant, loosen the pellet by tapping the bottom of the Falcon tube several times, and repeat the PBS wash (add 10 ml of PBS on the cells and centrifuge as in **step 7**). Loosen the pellet and add 1 ml of complete medium.
10. Count the cells (Peripheral Blood Mononuclear Cells [PBMCs]), which should total approximately 5 million.
11. Adjust the cell concentration to 5 million per ml (if needed, spin the cells again at 453 × *g* for 10 min and after having loosened the pellet, resuspend in an adequate amount of complete medium).
12. Place 4 µl of 130 nm Protamine–RNA particles (containing 1 µg of RNA and 1 µg of Protamine, obtained in **step 4** of Subheading 3.1) at the bottom of a well in a 96-well U bottom plate.
13. Add 200 µl of cells (one million) on top of the 4 µl of particles. As controls, prepare one well with 200 µl of cells alone, one well with 200 µl of cells and 1 µg of Protamine and one well with 200 µl of cells and 1 µg of RNA.

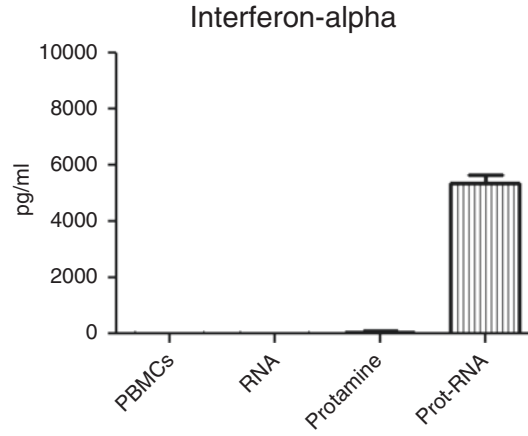

Fig. 2 Quantification of alpha interferons. The graph reports the calculated values of alpha-interferon content in supernatants from PBMCs alone ("PBMCs") or incubated with RNA ("RNA"), Protamine ("Protamine"), or Protamine–RNA nanoparticles approximately 130 nm in size (Prot-RNA). Each bar represents the average value of three data points (three culture wells) and the standard deviation

14. Incubate overnight at 37 °C in a humidified CO_2 incubator.
15. Transfer cell culture supernatants to a fresh 96-well plate. Proceed to **step 15** or store at −80 °C (*see* **Note 13**).
16. Use 20 µl of cell culture supernatant (add 80 µl per well of assay diluent: 1% BSA in PBS) in the pan-interferon-alpha ELISA kit strictly following the manufacturer's instructions (*see* **Note 14**).
17. Calculate experimental values. As shown in Fig. 2, Protamine–RNA nanoparticles of approximately 130 nm efficiently induce interferon-alpha expression in PBMCs in vitro (*see* **Notes 15** and **16**).

4 Notes

1. Protamine sulfate, such as Valeant Protamine, gave identical results.
2. We have observed that freezing Protamine solutions may jeopardize its functionality as far as production of immunostimulating RNA polyplex is concerned. Pharmaceutical Protamine should be stored at 4 °C as indicated on the package.
3. Any RNA (oligonucleotide, long uncapped and/or non-polyadenylated mRNA, or capped mRNA) can be used to generate the 130 nm particles.

4. Highly pure sterile RNA in water is very stable at room temperature. However, because contaminating RNases could theoretically be present, we store RNA at 4 °C (day storage) or −20 °C (long term storage) and bring it to room temperature when doing the experiments before immediately placing it back at 4 °C or −20 °C.
5. The more RNA and Protamine are diluted, the smaller the resulting particles will be (Supplementary Fig. 1a). Particles of 50–250 nm in size induce interferon-alpha in human blood cells in vitro to similar extents (Supplementary Fig. 1b).
6. Mixing equal mass amounts of Protamine and RNA will generate nearly neutral or slightly positive particles (zeta potential). Using a twofold mass excess of Protamine or a twofold mass excess of RNA will generate particles with positive or negative zeta potentials, respectively, as previously described [13]. Size (Supplementary Fig. 2a) and in vitro immunostimulation characteristics (Supplementary Fig. 2b) of the particles are similar when using particles with a positive, neutral, or negative surface charge within this range (twofold excess of one of the two components).
7. We always add Protamine to RNA, not RNA to Protamine.
8. This formulation is very stable at room temperature (as long as it is made using sterile RNA, Protamine, and water and in sterile conditions, i.e., under a laminar flow) or in the fridge. We have tested storage of this material up to 1 week and found that the storage period did not affect particle size or immunostimulatory capacity (Supplementary Fig. 3). By contrast, freezing the particles at −20 °C destroys their immunostimulatory capacity. Thus, it is of utmost importance to store the 130 nm Protamine–RNA nanoparticles in liquid solutions, and we recommend storage of undiluted particles at 4 °C.
9. We observed that the particles aggregate over time when diluted in salt-containing solutions, such as PBS or Ringer lactate, and thereby will appear larger than their original size if analyzed in those solutions. The original particle size is preserved when the particles are undiluted or diluted in water or other low-salt solution (e.g., isotonic 5% glucose). Thus, for particle size measurement, Protamine–RNA nanoparticles must be preserved by dilution in water or other low-salt solution.
10. A PDI value of 1 indicates that the sample has a very broad size distribution and may contain large particles or aggregates that could be slowly sedimenting. A PDI value closer to zero denotes a monodispersed system (one unique particle size in the sample). For biological particles, it is usually accepted that a PDI value below 0.5 represents a relatively homogenous formulation.

11. Blood stored up to 24 h at 4 °C can be used. However, frozen blood cells did not respond as well as did fresh or stored (up to 24 h) blood. We suspect that plasmacytoid dendritic cells, an important producer of interferon-alpha, do not survive freeze–thaw cycles well when cells are frozen in physiologic solutions containing 10% DMSO.
12. The centrifuge should accelerate and decelerate as slowly as possible. Should acceleration or deceleration be too fast, the interface between blood and Ficoll will be perturbated, and the collection of peripheral blood mononuclear cells will be jeopardized. Should this happen (for example, by mistake the brake is not disabled during centrifugation), the blood and Ficoll can be manually further mixed together (inverting the tube few times) and 5 ml Ficoll can again be underlay with 5 ml of Ficoll as in **step 3** of Subheading 3.3 before centrifugation under the appropriate conditions.
13. We observed that supernatants stored at −20 °C were not well preserved as far as interferon-alpha is concerned.
14. Using 20 μl of cell culture supernatants usually allows experimental ELISA optical densities (ODs) that are in the range of the standard titration (from 0 to 1000 pg/ml).
15. The total amount of alpha interferon detected in supernatants varies from donor to donor and can be in the range of 1000–10,000 pg/ml.
16. For each condition, we performed three PBMC cultures (triplicates) and presented the data as the means plus standard deviation.

Acknowledgement

The research leading to these results has received funding from the European Union's Seventh Framework Programme (FP7/2007-2013) under grant agreement n° 601939 (MERIT).

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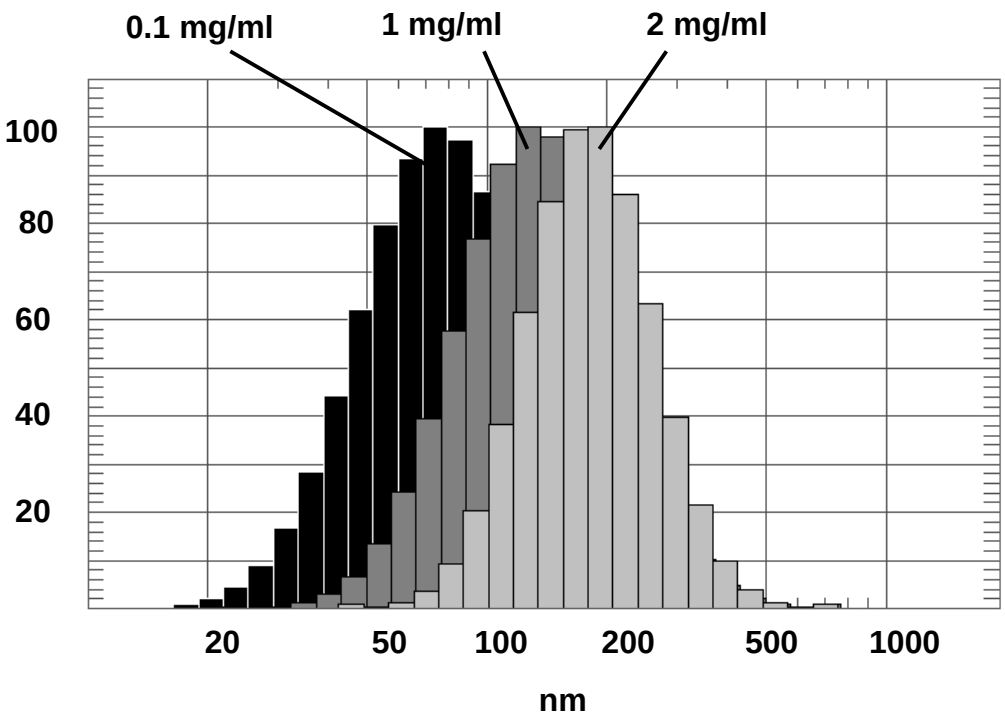
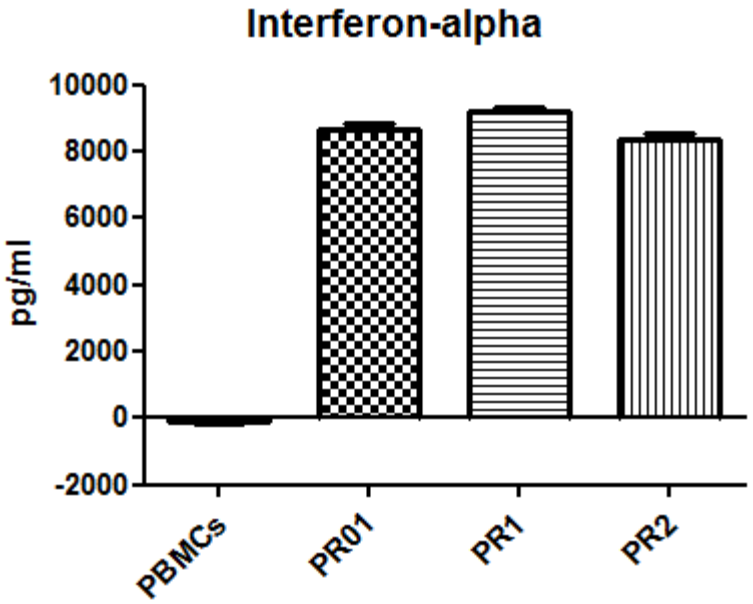
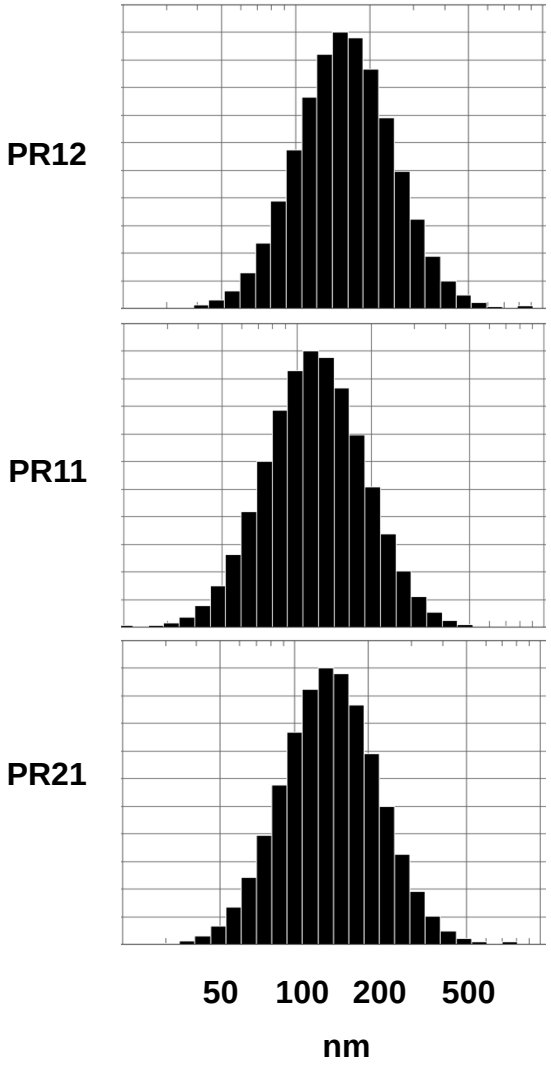
Supplementary Figure 1A



Supplementary Figure 1B



Supplementary Figure 2A



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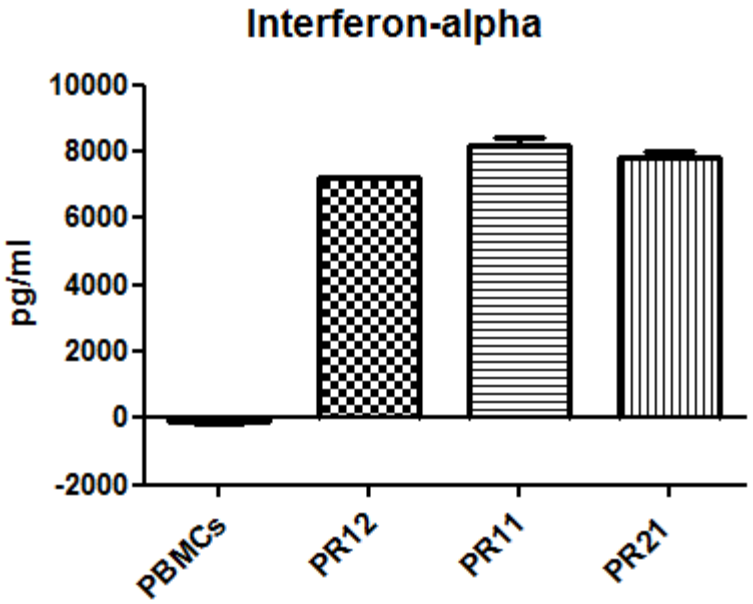
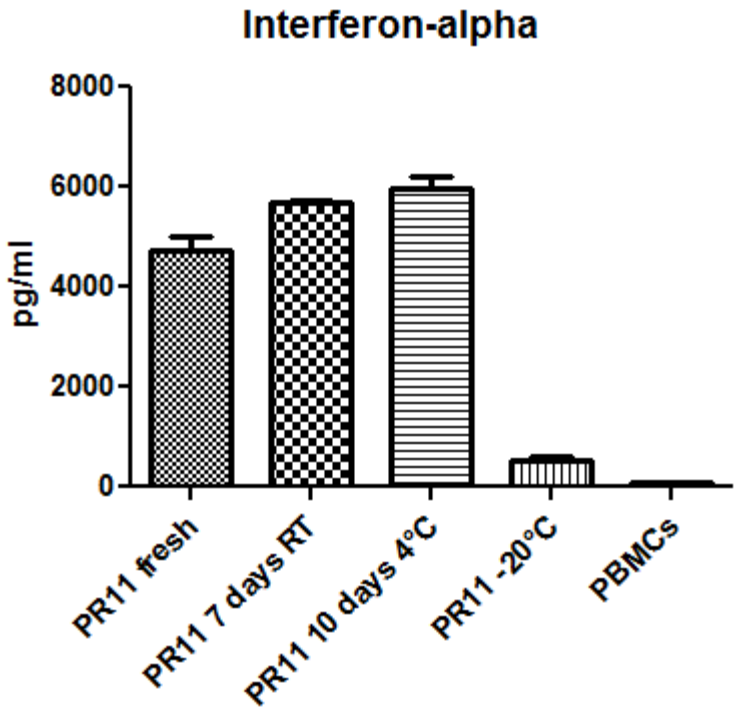


Supplementary Figure 3



Supplementary Fig. 1 (**a**) Influence of reagent dilutions: Particle size. Protamine and RNA were diluted in water at 0.1, 1, or 2 mg/ml before being mixed together (mass ratio 1:1). The graphs report "Intensity-Weighted Gaussian Distribution Analysis" for solid particles. Average particle sizes are 85 nm for 0.1 mg/ml, 146 nm for 1 mg/ml, and 192 nm for 2 mg/ml. Thus, particle size can be finely tuned by adjusting the Protamine and RNA ratio. The greater the dilution, the smaller the particles. (**b**) Influence of reagent dilutions: Induction of alpha interferons. The graph reports the calculated values of alpha-interferon contents in supernatants from PBMCs alone ("PBMCs") or those incubated with Protamine–RNA nanoparticles made with reagents at 0.1 mg/ml ("PR01"), 1 mg/ml ("PR1"), or 2 mg/ml ("PR2"). Each bar represents the average value of three data points (three culture wells) and the standard deviation. The three types of particles induce similar amounts of alpha interferons.

Supplementary Fig. 2 (**a**) Influence of reagent ratio: Particle size. Protamine and RNA were diluted in water at 0.5 mg/ml before being mixed together at mass ratios of 1:2 (twofold more RNA than Protamine "PR12"), 1:1 (equivalent mass amounts of RNA and Protamine "PR11"), or 2:1 (twofold more Protamine than RNA "PR21"). The graphs report "Intensity-Weighted Gaussian Distribution Analysis" for solid particles. Average particle size is 174.7 nm for the 1:2 ratio, 131.6 nm for the 1:1 ratio, and 154.7 nm for the 2:1 ratio. Thus, particle sizes are similar when using Protamine–RNA ratios from 1:2 to 2:1. (**b**) Influence of reagent ratio: Induction of alpha interferons. The graph reports the calculated values of alpha-interferon content in supernatants from PBMCs alone ("PBMCs") or those incubated with Protamine–RNA nanoparticles made with reagents at a 1:2 mass ratio ("PR12"), 1:1 mass ratio ("PR11") or 2:1 mass ratio ("PR21"). Each bar represents the average value of three data points (three culture wells) and the standard deviation. The three types of particles induce similar amounts of alpha interferons.

Supplementary Fig. 3 Stability of particles: Induction of alpha interferons. The graph reports the calculated values of alpha-interferon content in supernatants from PBMCs alone ("PBMCs") or those incubated with Protamine–RNA nanoparticles made with reagents at a 1:1 mass ratio and used immediately ("PR11 fresh") as well as after storage at room temperature for 1 week ("PR11 7 days RT") or 4 °C for 10 days, ("PR11 10 days 4 °C") or after having been frozen and thawed once ("PR11 –20 °C"). Each bar represents the average value of three data points (three culture wells) and the standard deviation. The particles are very stable when kept in liquid but partly deactivated by freezing/thawing.

Cancer Chemother Pharmacol
DOI 10.1007/s00280-017-3446-y

ORIGINAL ARTICLE

Phase I study of a chloroquine–gemcitabine combination in patients with metastatic or unresectable pancreatic cancer

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Received: 3 May 2017 / Accepted: 23 September 2017

Abstract
Purpose Following a previously published pre-clinical validation, this phase I study evaluated the safety, maximum tolerated dose, anti-tumour activity and immune status of a gemcitabine–chloroquine combination as a first- or late-line treatment in patients with metastatic or unresectable pancreatic cancer.
Methods In this 3 + 3 dose escalation study, patients received a single weekly standard dose of intravenous gemcitabine, followed by single weekly oral intake of 100, 200 or 300 mg of chloroquine. Tumour response was assessed using the Response Evaluation Criteria in Solid Tumors version 1.1. Immune status was evaluated by RT-PCR to measure the relative expression of immune-related genes in peripheral blood mononuclear cells (PBMCs).
Results Overall, nine patients [median age 72 years; interquartile range (IQR), 68–78 years] were treated. No dose-limiting toxicities as defined in the protocol were observed. Three patients experienced partial response, and two patients had stable disease. The median time to progression was 4 months (95% CI 0.8–7.2), and the median overall survival was 7.6 months (95% CI 5.3–9.9). Among 86 assayed immune genes, three were significantly differentially expressed in PBMCs from responding versus non-responding patients: interferon-gamma receptor-1, toll-like receptor 2, and beta-2 microglobulin.
Conclusions The addition of chloroquine to gemcitabine was well tolerated and showed promising effects on the clinical response to the anti-cancer chemotherapy. Based on these initial results, the efficacy of the gemcitabine–chloroquine combination should be further assessed.

Keywords Chloroquine · Gemcitabine · Pancreatic cancer · Interferon-gamma receptor-1 · Toll-like receptor 2 · Beta-2 microglobulin

Electronic supplementary material The online version of this article (doi:10.1007/s00280-017-3446-y) contains supplementary material, which is available to authorized users.

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Introduction

Pancreatic adenocarcinoma is the fourth most common cause of cancer-related death, and it has a poor prognosis: for all stages combined, the 5-year relative survival rate is 8%; for local disease, the 5-year survival is approximately 29% [1]. The median survival for locally advanced and metastatic disease, which collectively represents more than 80% of individual cases, is approximately 10 and 6 months, respectively. In patients with locally advanced or metastatic pancreatic cancer, chemotherapy with gemcitabine alone at a weekly (3

Published online: 04 October 2017

of 4 weeks) dose of 1000 mg/m^2 is widely used to improve patient's quality of life with a modest survival benefit [2]. Furthermore, a median overall survival of 11.1 months can be achieved with FOLFIRINOX (5-fluorouracil, leucovorin, irinotecan, oxaliplatin), in contrast to 6.8 months using weekly gemcitabine treatment [3]. However, significantly more adverse events were noted in the FOLFIRINOX group, highlighting the need to carefully select patients for this more intensive protocol. More recently, a weekly gemcitabine-based combination with albumin-bound paclitaxel (nab-paclitaxel, 3 of 4 weeks) also resulted in improved outcomes compared to gemcitabine alone in patients with advanced pancreatic cancer, thus establishing the former regimen as an additional option in patients with advanced disease. The median overall survival was improved from 6.7 months with gemcitabine alone to 8.5 months with the combination treatment. Relevant adverse events observed with this combination treatment included fatigue, peripheral neuropathy and diarrhoea [4].

Chloroquine has been a widely used prophylactic (300 mg per week) and therapeutic (300 mg daily) anti-malarial drug for more than 60 years [5]. More recently, chloroquine was shown to be an immunomodulator [6] and demonstrated anti-cancer activities in vitro and in pre-clinical models [7]. Chloroquine increases the endosomal pH and has opposing effects depending on the dose and schedule [7]. For example, chloroquine helps control autoimmune diseases (lupus erythaematosus and rheumatoid arthritis) when administered at high daily doses (300 mg daily), whereas a single dose of 300 mg of chloroquine administered 2 days before a protein vaccine enhances the induction of T cells [8]. In addition, chloroquine has several anti-tumour activities in vitro and in vivo, either by having a direct effect on cancer (stem) cells or by modifying the tumour microenvironment [7, 9]. Daily uptake of chloroquine combined with chemotherapy (carmustine) and radiotherapy was found to enhance the overall survival of patients with glioblastoma (post-surgical tumour ablation) [10, 11]. Similarly, previous work in our laboratory showed that in mouse tumour models, the efficacy of gemcitabine is improved by the administration of chloroquine [12]. Importantly, the single administration of chloroquine must follow, not precede, the single injection of gemcitabine to enhance the control rate of established tumours in animal models. However, when tested as an anti-cancer drug, chloroquine was usually added daily (before, during and after radiochemotherapies [10, 11]), without preliminary evaluation of the potential opposite effects of the anti-cancer treatments and chloroquine. Certain chemotherapeutic drugs, such as gemcitabine, belong to a group of immunologic cell death inducers and contribute to the induction of anti-cancer immunity by dying tumour cells [13]. This induced immune response helps control the disease. It was previously postulated by our laboratory that chloroquine administered after gemcitabine further induces the death of tumour cells (including tumour stem cells) without jeopardizing anti-cancer immunity [12]. A phase I study was, therefore, implemented with the objective of evaluating the safety, tolerability and preliminary efficacy of increasing doses of chloroquine given 1 day after weekly gemcitabine treatment in patients with locally advanced or metastatic pancreatic cancer. An additional objective was to analyse peripheral blood to identify immune markers that would correlate with treatment response.

Materials and methods

Patient population

Patients 18 years or older with a WHO performance status of 0–2 and histologically or cytologically confirmed non-resectable locally advanced or metastatic adenocarcinoma of the pancreas were eligible. Adequate liver and kidney function tests [bilirubin < 2× upper limit of normal (ULN), alanine aminotransferase (ALT) < 5× ULN, alkaline phosphatase < 5× ULN, estimated creatinine clearance > 40 ml/min using the Cockcroft formula], and adequate haematological values (haemoglobin > 80 g/l, leucocytes > 3.0 G/l, neutrophils > 1.0 G/l, platelets > 100 G/l) were mandatory prior to inclusion in the study. Previous treatment with chemotherapy, including gemcitabine, and/or radiotherapy was not an exclusion criterion.

Study design

ONK-USZ-004 is a single-arm monocentric phase I study sponsored by the University Hospital of Zürich. The study protocol and amendments were approved by the local Ethics Committee and Swissmedic (ClinicalTrials.gov identifier: NCT01777477). Each patient provided informed consent. Although the protocol was initially written for exclusive recruitment of treatment-naïve patients, an amendment was filed in April 2014 to permit the recruitment of previously treated patients. Dose escalation was conducted using a traditional 3 + 3 algorithm, with cohorts of three patients who were sequentially enrolled at chloroquine single doses of 100 mg (the lowest dose used for prophylaxis of malaria: 1 tablet), 200 and 300 mg (the highest recommended daily dose) total per week (on days 2, 9, and 16 of a 4-week cycle repeated on day 30) orally 1 day after gemcitabine administration (1000 mg/m^2 i.v. on days 1, 8 and 15 of a 4-week cycle; repeated on day 29). Treatment was performed until progression or unacceptable toxicity was observed or until the patient requested treatment termination. Dose-limiting toxicity (DLT) was defined as an adverse event (AE) or laboratory abnormality that fulfilled the following criteria

during the first cycle of systemic treatment: grade 4 neutropenia lasting at least 7 days or febrile neutropenia; grade 4 thrombocytopenia; grade 3/4 gastrointestinal symptoms (diarrhoea, vomiting or mucositis) that were not reduced to grade 1 within 7 days of appropriate supportive care; grade 3/4 skin toxicity (e.g. rash or hand-foot-syndrome); grade 3/4 ocular toxicity (e.g. blurred vision or retinopathy); grade 3/4 auditory toxicity (e.g. hearing loss or tinnitus); grade 3/4 neurologic toxicity; grade 3/4 pulmonary toxicity (e.g. dyspnoea, oedema or alveolitis) that did not resolve to grade 1 within 3 days after treatment cessation; grade 3/4 elevation of liver transaminases and bilirubin, despite discontinuation of treatment and exclusion of stent failure or hepatic tumour progression; and any grade 3/4 toxicity that was considered dose limiting by the principal investigator. Adverse events that were determined by the investigator to be unrelated or remotely related to the experimental drug (chloroquine) were not considered in the decision of whether to escalate the dose in the next cohort. The maximum tolerated dose (MTD) of chloroquine in combination with the fixed dose of gemcitabine was defined as one dose level below the dose level that caused DLTs in at least two patients. No further dose escalation of chloroquine above 300 mg per week was pursued because higher doses may cause immunosuppression [14, 15] and increased incidence of retinopathy.

Study endpoints

The MTD of orally administered chloroquine combined with intravenous gemcitabine was the primary endpoint. The secondary endpoints were response rate according to RECIST v1.1, best response under trial treatment (assessed every 2 months), progression-free survival and overall survival.

Since gemcitabine and chloroquine are immune-related drugs, immune assessments of the patients were also performed.

Assessments

Adverse events were graded according to the National Cancer Institute Common Terminology Criteria for Adverse Events (NCI-CTCAE) v4.0 http://evs.nci.nih.gov/ftp1/CTCAE/CTCAE_4.03_2010-06-14_QuickReference_5x7.pdf). Tumour response was assessed by the investigator every 2 months during therapy and every 3 months thereafter by computed tomography (CT) scans of the chest, abdomen, or pelvis.

Immunomonitoring

For each patient, 6 lithium heparin tubes (approximately 50 ml in total) and 1 serum blood sample (approximately 10 ml) were collected prior to treatment initiation and at every other cycle during therapy, including once at the first follow-up visit. Peripheral blood mononuclear cells (PBMCs) were recovered from heparin tubes by centrifugation in a Ficoll gradient and then were frozen in foetal calf serum containing 10% DMSO (up to 10 million cells per aliquot). For analysis, one aliquot was thawed, and RNA was extracted using the Qiagen miRNA Kit according to the manufacturer's instructions. The RNA was then reverse transcribed and used in the Qiagen RT2 profiler according to the manufacturer's instructions.

Results

Patients

Between October 2012 and February 2015, nine patients (four female and five male patients) were enrolled in the study (Table 1). The median age of the patients was 72 years [interquartile range (IQR), 68–78 years]. All patients had symptomatic pancreatic cancer at study entry (e.g. pain, fatigue, anorexia, and weight loss). One patient underwent resection of the primary tumour at diagnosis. This patient had an additional small neuroendocrine tumour of the pancreatic tail (13 mm in diameter, G1, MIB < 2%), which had been completely excised at primary resection. After discussion with the study team, the patient was considered eligible despite the underlying additional low-grade malignancy. Two patients received anti-cancer treatment before study entry: one patient was treated with radiotherapy in combination with capecitabine for locally advanced disease, and the other patient received gemcitabine monotherapy for metastatic disease. All patients received at least two subsequent weekly infusions of gemcitabine followed by chloroquine 1 day later and were thus evaluable. Two patients had locally advanced inoperable pancreatic cancer, and all other patients had metastatic disease.

Dose modifications and safety

A total of 39 cycles were administered. Gemcitabine was administered at 110 (94%) of the planned 117 therapy visits, with subsequent intake of chloroquine the day after in 109 (93%) of these visits: the planned infusion of gemcitabine on day 15 and the subsequent chloroquine intake on day 16 of the final treatment cycle were omitted in six patients. Additional administrations of gemcitabine and chloroquine on days 15 and 16, respectively, of the second to last cycle were also omitted in one patient. Thrombocytopenia induced by gemcitabine led to a dose reduction of the chemotherapeutic drug on day 8 or 15 in 13 of the total of 110 (12%) gemcitabine infusions without reduction of the subsequent chloroquine dose.

Table 1 Patient characteristics

Chloroquine single dose	#	Age	Sex	WHO PS	Tumour location	Tumour extent	Metastatic sites at baseline	Previous treatment	Relevant pre-existing conditions
100 mg	1	73	Female	1	Tail	Metastatic	LN, liver	–	Venous sinus thrombosis; poliomyelitis; apoplexy; hypertension
	2	72	Female	2	Head	Metastatic	LN, liver, lung	–	Fatigue; recurrent erysipelas; metabolic syndrome
	3	78	Male	1	Tail	Metastatic	LN, peritoneum, lung, bone	–	Deep vein thrombosis with pulmonary embolism; fatigue
200 mg	4	63	Male	1	Head	Locally advanced	None	Radiotherapy plus Capecitabine	Pancreatic fistula with pleural effusion; duodenal stenosis; diabetes mellitus
	5	70	Female	0	Head/body	Locally advanced	None	–	Neuropathy; renal insufficiency; hypertension
	6	82	Male	1	Body/tail	Metastatic	LN, liver, peritoneum	Resection of the primary tumour	Pulmonary hypertension; deep vein thrombosis; ascites
300 mg	7	63	Male	2	Head	Metastatic	Lung, peritoneum	–	Portal vein thrombosis with portal hypertension; occlusion of mesenteric and splenic vein; congenital heart valve disease
	8	68	Female	0	Tail	Metastatic	LN, liver, peritoneum	–	
	9	82	Male	0	Head	Metastatic	LN, liver	Gemcitabine	Fatigue; hypertension

LN lymph nodes, *WHO PS* World Health Organization Performance Score Status

Most adverse events observed were expected according to the underlying disease and the administration of gemcitabine. Five episodes of temporary grade 1/2 vision impairment (blurred vision) observed in two patients were considered possibly related to chloroquine and did not result in treatment changes. All adverse events ≥ grade 3 are listed in Table 2. The complete list of adverse events is available as Supplementary Table 1.

No DLTs as defined in the protocol were observed, and the number of patients did not need to be increased in any cohort. Significant overlapping toxicities of the additional intake of chloroquine were not observed up to the highest dose evaluated of 300 mg once per week.

Clinical outcomes

The objective tumour responses according to RECIST criteria are shown in Table 3. Overall, three patients (33%) experienced partial responses, and two patients (22%) had stable disease. One patient decided to stop therapy after two cycles despite disease stabilization and subsequently received a dose-reduced FOLFIRINOX regimen. Therefore, the analysis of time to progression was performed as intention to treat and as per protocol after censoring this patient at the time of new treatment initiation.

Disease stabilization was reached as early as 1.9 months after treatment start and lasted up to 7.9 months

Table 2 Total number of observed adverse events ≥ grade 3, regardless of relationship to treatment, reported during treatment (39 cycles administered overall) or during follow-up of the nine patients

Adverse event	Dose level 1 (100 mg)		Dose level 2 (200 mg)		Dose level 3 (300 mg)	
	Grade 3	Grade 4	Grade 3	Grade 4	Grade 3	Grade 4
Haematologic events						
Anaemia			2		1	
Leukocytopenia	2		1		2	
Neutropenia	4		1		2	
Lymphocytopenia	1		2		2	
Thrombocytopenia			2	1	1	
Non-haematologic events						
Diarrhoea			1		1	
Nausea/vomiting			2			
Fatigue/weakness			1		1	
Anorexia/weight loss			1		2	
Pain	1		1		1	
Bile duct/duodenal/urinary tract compression	1		2		1	
Infection, any	1		3		1	
Peritoneal effusion			1		1	
Secondary malignancy			1			
ASAT/ALAT elevation	1		2			
AP/GGT elevation		1	1			
Hypoalbuminaemia			2			
Hyponatraemia			1			
Hypokalaemia	2	1	1			

Only pre-existing conditions that worsened during or after treatment are reported. The highest grade observed of each AE is reported

Table 3 Objective response rates

Chloroquine dose	Patient #	Month 2	Month 4	Month 6	Month 8	Month 9	Cycles administered
100 mg	1	SD (− 27%)	PR (− 39%)	PR (− 35%)			6
	2	PR (− 39%)	PD (− 11%; +<u>46%</u>)				4
	3	SD (− 16%)					2
200 mg	4	SD (− 20%)	PR (− 39%)	PR (− 45%)	PR (− 45%)		6
	5	SD (+12%)	SD (− 11%)	SD (− 6%)	SD (− 11%)	SD (+2%)	11
	6	PD (− 24% but new lesions)					2
300 mg	7	PD (− 3% but new lesions)					5
	8	PD (+9 plus new lesions)					2
	9	PD (clinical assessment)					1

The percent increase or decrease of the total tumour volume compared to baseline is shown in brackets. In addition, the increase of the tumour volume compared to the nadir is shown underlined in patient #2, indicating progressive disease

PR partial response, *SD* stable disease, *PD* progressive disease

in one patient. Response was achieved after 1.8 months in one and after 3.9 months in two patients. Response duration lasted between 2.2 and 6.9 months. The median time from treatment start to tumour progression was 4 months (95% CI 0.8–7.2) in the intention to treat analysis and 4 months (95% CI 0.9–7.1) as per protocol. The

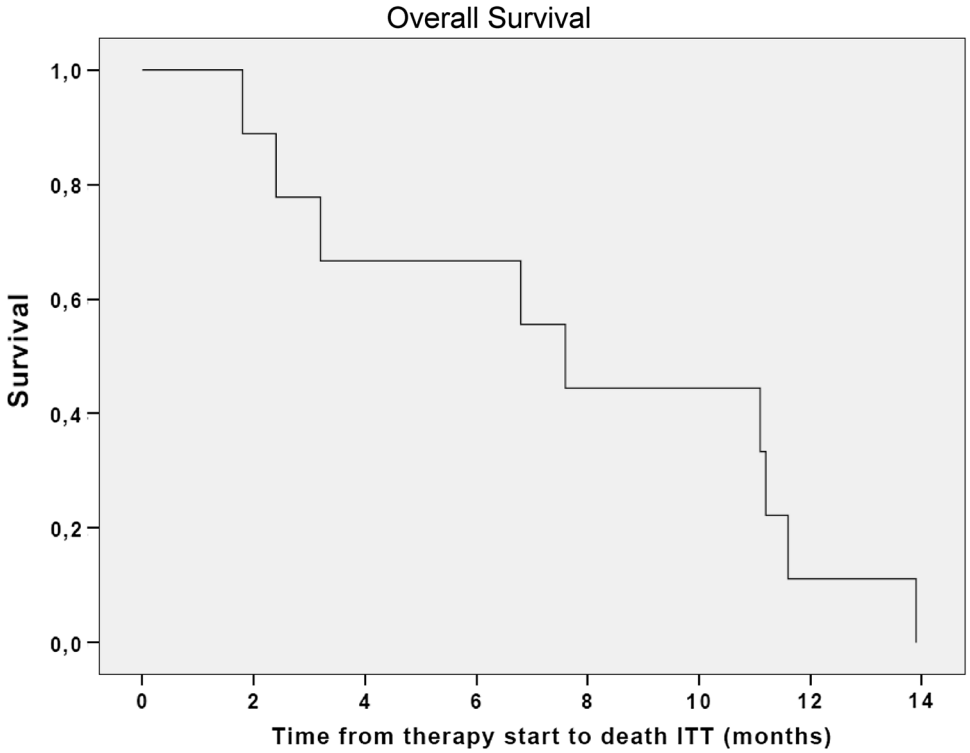

Fig. 1 Kaplan–Meier analysis of the overall survival of the patients. The median overall survival was 7.6 months (95% CI 5.3–9.9)

median overall survival was 7.6 months (95% CI 5.3–9.9) (Fig. 1).

Immune status

Gemcitabine is an immunologic cell death inducer, and thus the PBMCs of patients were analysed at baseline to identify correlations between immune status and clinical outcome after gemcitabine/chloroquine treatments. To obtain a broad picture of the immune status, the expression levels of 81 immune-related genes (innate and adaptive immunity) in PBMCs were measured by real-time PCR with the Qiagen RT2 system. The analyses were compared among three patients with an objective clinical response (partial response according to RECIST was observed in patients #1, #2 and #4) and patients with progressive disease (only patients #6, #7 and #9 were used because the quality of RNA extracted from the PBMCs of patient #8 was not adequate to perform the RT2 assay). After normalization to the beta actin expression levels, three of the 81 tested genes were significantly differentially expressed between these two patient groups: interferon-gamma receptor-1 (IFNGR1) and toll-like receptor-2 (TLR2) were decreased in responding patients compared with non-responding patients, whereas beta-2 microglobulin (B2M) was increased in responding patients compared with non-responding patients (Fig. 2). The data for all genes are reported in Supplementary Table 2.

Discussion

Chloroquine, a drug that diffuses freely and rapidly across cell membranes and enhances endosomal pH, has a plethora of biological effects that are dose/schedule dependent and may be useful against cancer [7], including the induction of apoptosis, vacuolization, inhibition of autophagy, inhibition of the multiple resistance pump (MRP), buffering of tumour milieu (for improved biodistribution of charged chemotherapeutic drugs), interaction with nucleotides, toxicity in cancer stem cells, normalization of the vasculature, and modulation of the immune response. In mice, the administration of chloroquine after intraperitoneal infusion of gemcitabine enhances the control of tumour growth, although the exact underlying mechanisms have not been elucidated [12]. Gemcitabine is an immunological death inducer [13], and its anti-cancer activity is partially attributed to the stimulation of innate immunity by dead cells and the induction of an anti-cancer adaptive immune response. Thus, the efficacy of gemcitabine requires a functional immune system and may be limited when used in combination with an immunosuppressive treatment, such as 300 mg daily chloroquine. The present phase 1 dose escalation trial was conducted to evaluate the safety of chloroquine when combined with gemcitabine and to assess preliminary data on the efficacy of this chemotherapeutic combination in a selected patient population with pancreatic cancer. Nine patients diagnosed with locally advanced or metastatic pancreatic cancer were recruited at three chloroquine doses following the classical

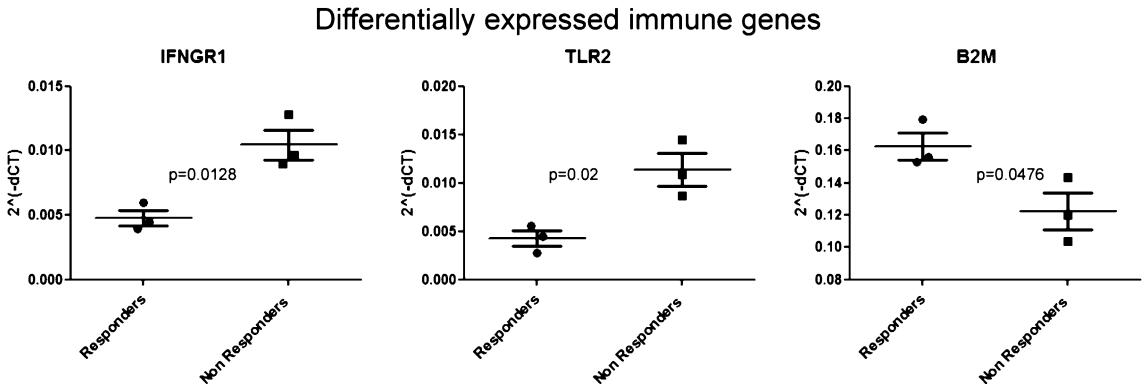

Fig. 2 Differential expression of immune genes between responders and non-responders. PBMCs obtained from the patients immediately prior to the first infusion of gemcitabine were frozen in liquid nitrogen. Total RNA from thawed cells was used for real-time PCR with the Qiagen RT2 Innate and Adaptive Immunity Kit. Responding patients (defined as a partial response according to the RECIST criteria) were #1, #2 and #4. Non-responding patients (defined as having progressive disease according to RECIST) were #6, #7 and #9. The relative expression levels were calculated using the following formula: $2^{-(\text{CTgene\_of\_interest}-\text{CT beta\_actin})}$. Long horizontal lines and short horizontal lines indicate the means and standard errors of the mean, respectively. The *p* value between responders and non-responders was calculated using a two-tailed unpaired *t* test

3 + 3 enrolment rule (Table 1). In total, more than 100 infusions of gemcitabine plus 100/200/300 mg chloroquine were administered, according to the respective cohort.

Excess toxicity with increasing doses of chloroquine added to gemcitabine was not observed among the patients in this study. The documented adverse events (Table 2) were predominantly related to the health status of the patients or were anticipated during gemcitabine treatment. Omission of gemcitabine infusion at day 15 is frequent in daily practice outside clinical trials. Such omissions are usually the result of higher grade thrombocytopenias or granulocytopenias, which are typical adverse events of gemcitabine observed in up to 25% of patients, even when administered as monotherapy. Accordingly, the need for treatment omission was not unusual within this trial. Thus, the highest evaluated chloroquine dose of 300 mg per week combined with standard dosed gemcitabine was feasible. No DLT and no grade 4 toxicities were observed in this cohort.

Three of nine patients showed a partial response, and two patients had stable disease according to the RECIST criteria for an overall response rate of 33% and a tumour control rate of 55%. This finding compares favourably to the previously reported and anticipated response rates of up to 10% with gemcitabine alone in patients with advanced pancreatic cancer [2–4]. Responses were observed in the groups receiving lower doses of chloroquine (100 or 200 mg), suggesting that, as indicated in our pre-clinical studies [12], lower doses of chloroquine may be more efficacious in this treatment setting. However, as only nine rather elderly patients were treated, the outcome analysis may only be considered exploratory.

Evaluation of the global immune status of the patients based on expression profiling of 80 immune-related genes in PBMCs obtained immediately before the start of therapy revealed that three genes may predict the response to therapy: relatively low expression of IFNGR1 and TLR2 or relatively high expression of B2M. Indeed, it has been reported that the gamma-interferon axis may be tumour promoting by inducing immunosuppressive effectors (e.g. indoleamine-2,3-dioxygenase or PDL1 [16]). The interim analysis of a randomized phase III trial showed statistically shorter overall survival in cancer patients who received IFN-γ 1b plus paclitaxel/carboplatin compared to patients who received paclitaxel/carboplatin alone [17]. This decrease in overall survival and increase in treatment-limiting AEs led to the termination of this trial at the second interim analysis. Thus, interferon-gamma is a double-edged sword, and a relatively decreased susceptibility to interferon-gamma through lower IFNGR1 expression may increase the anti-cancer immunity required for the full efficacy of gemcitabine. A similar association between high expression and unresponsiveness to therapy has been reported for TLR2, the second gene identified in this study as overexpressed in non-responding versus responding patients. Specifically, Yang et al. reported that the persistent activation of TLR2 by heat-shock protein 60 by tumour cells led to the release of immunosuppressive cytokines and chemokines [18]. Accordingly, TLR2 deficiency protected mice from the progression of B16 melanoma, and combining an anti-TLR2 antibody with gemcitabine improved the survival of wild-type tumour-bearing mice. Responding patients showed a significantly higher expression of B2M. Because this protein is involved in antigen presentation to cytotoxic T cells, its expression is needed to educate lymphocytes and promote T cell-mediated killing of their target cells. Thus, it could be postulated that high expression of B2M predisposes patients to an improved

cytotoxic T cell response. The confirmation of a favourable baseline signature based on the expression of IFNGR1, TLR2 and B2M would require the analysis of PBMCs from more patients in a subsequent trial. These immune parameters were not studied during treatment because the drugs given to the patients, particularly gemcitabine and chloroquine, modify the immune system.

In conclusion, the results of this study demonstrate that the gemcitabine–chloroquine regimen is well tolerated, especially in elderly patients with advanced pancreatic cancer, and may induce promising clinical responses. Immune parameters, such as a low expression of IFNGR1 and TLR2 and high expression of B2M, may predispose patients to a better response to this combination treatment. Furthermore, adding chloroquine to the administration of chemotherapeutic drug(s), mostly those inducing immunological cell death, may be a safe way to enhance clinical responses. The optimal timing and dosing of chloroquine must be defined through pre-clinical studies; however, the appropriate administration of chloroquine (time and dose) as presented here may be a safe adjuvant to approved anti-cancer treatments. Based on these data, the efficacy of chloroquine added to gemcitabine monotherapy or combination regimens should be evaluated.

Acknowledgements This work was supported by the Julius Müller Stiftung and the Kurt und Senta Hermann Stiftung.

Compliance with ethical standards

Funding This study was funded by the University Hospital of Zürich, the Julius Müller Stiftung and the Kurt und Senta Hermann Stiftung.

Conflict of interest Authors Samaras, Nguyen-Kim, Seifert, Bachmann, Knuth and Pascolo declare that they have no conflict of interest. Author von Moos is a participant in an advisory board of Elli Lilly.

Animals This article does not contain any studies with animals performed by any of the authors.

Ethical approval All procedures performed in this study involving human participants were in accordance with the local Ethics Committee and Swissmedic (ClinicalTrials.gov identifier: NCT01777477) and with the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

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7. Conclusion and outlook

Presence of diverse posttranscriptional RNA modifications in natural RNA provides an additional level of complexity in RNA biology. RNA functionality was mainly attributed to its sequence information. However, advances in identification and localization of nucleotide modifications started to disclose new functional roles (Dominissini et al., 2012; Safra et al., 2017; Schwartz et al., 2014a). In particular, studies using lipofectin-formulated modified synthetic RNA or subpopulations of RNA showed presence of these modifications suppresses recognition by specific receptor of the innate immunity and underlined their relevance in self- versus non-self RNA recognition (Kariko et al., 2005).

Research on m6A and m1A provided a novel role of modifications indicating that these RNA markings are rather dynamic than static (Dominissini et al., 2016; Jia et al., 2012). Studies of the enzymatic machineries introducing, reading and removing m6A modifications opened a field of research for regulatory networks guiding these reactions. Moreover, single nucleotide mapping of m6A, m1A, 2′-O-methylation and Ψ showed that these modifications are abundant in mRNA and non-coding fractions other than rRNA and tRNA: lncRNA, miRNA, snRNA and snoRNA (Carlile et al., 2015; Dominissini et al., 2012; Dominissini et al., 2016; Krogh et al., 2017; Li et al., 2016b; Meyer et al., 2012; Schwartz et al., 2014a). Localizing and mapping these modifications in the complete population of mRNA found in a cell constitutes the epitranscriptome (Grozhik and Jaffrey, 2017). It has been shown that m6A differentially affects translation, alternative splicing, polyadenylation, and nuclear export of mature mRNA depending on its position the molecule (Fustin et al., 2013; Ke et al., 2015; Liu et al., 2015; Liu et al., 2017; Meyer et al., 2015; Zhou et al., 2015). Possessing all these roles suggested m6A and possibly other modifications act as molecular switches linking environmental changes and prompted cellular responses.

Summarizing the known roles of the m6A, inosine, 2′-O-methylation and Ψ epitranscriptome in cancer we highlight the possibility to take into consideration epitranscriptomics in future diagnosis and treatment of cancer. In addition, we show that a chemotherapeutic drug known to block methylation of nucleic acids is changing the immunostimulating profile triggered by RNA.

capable of changing RNA modifications. Pentostatin was identified over thirty years ago as a chemotherapy efficacious for the treatment of specific leukemias and lymphomas (Braiteh et al.,

2006; Dillman et al., 1988; Dohner et al., 1993; Grever et al., 1983; Spiers et al., 1984). Surprisingly, it is not toxic on cancer cells *in vitro* (Wilson et al., 1998).

Studying innate immune responses triggered by total RNA from organisms of different evolutionary complexity we found that Protamine-RNA nanoparticles are able to unravel the immunostimulatory potential of mammalian RNA. Namely, we demonstrated that total RNA from lower but also higher organisms when formulated in Protamine induce TNF-α production in hPBMCs. This contradicted previous findings where only RNA from lower organism was found to be immunostimulating. This discrepancy is possibly due to differences in formulations. Protamine-RNA nanoparticles may deliver RNA preferentially to the endosomes while liposomal formulations may deliver RNA preferentially to the cytosol resulting in different spatial distribution and sensing by segregated immune receptors. Since RNA-recognizing TLRs are in the endosomes, the induction of TNF-α that we observe with Protamine-RNA was most probably missed in studies using lipofectin as transfecting agent.

Through further characterizing the immunostimulation profiles induced by natural RNA we could show that even heavily modified mammalian RNA induces not only TNF-α but several other cytokines in hPBMCs. However, IFN-α was induced exclusively by total RNA isolated from lower organisms. Knowing that RNA from higher organisms bears more modifications compared to RNA from lower organisms, we hypothesized that the presence of specific RNA modifications is responsible for blocking IFN-α induction by total mammalian RNA. Our own experiments and the literature pointed to a possible role of adenosine to inosine editing and methylations in masking RNA from immune sensors. Therefore, we tested drugs that inhibit adenosine deaminases responsible for maintenance of balanced methylation index and inosine level in cells (Glazer and Hartman, 1980; Johnston and Kredich, 1979; Kredich, 1980). From all drugs known to directly or indirectly block adenosine deaminases only Pentostatin, the most potent inhibitor, showed to enhance triggering of type I interferon by mammalian RNA *in vitro* and *in vivo*. In the early eighties it was already shown that Pentostatin affects 2′-O-methylation in RNA (Hershfield et al., 1983). 2′-O-methylation was evidenced to block recognition of RNA by TLR3 and TLR7 in human cells (De Clercq et al., 1972; Jung et al., 2015). Of note, recombinant alpha interferon was used as a treatment against hairy cell leukemia before introduction of Pentostatin (Golomb et al., 1986). We were able to associate this previous knowledge with our findings and show that Pentostatin

although not toxic on cancer cells *in vitro* is efficacious in the treatment of tumor bearing mice via dsRNA sensing by TLR3 and resulting type I interferon induction. All these findings re-purpose Pentostatin as an immunomodulating drug having potential in cancer treatment alone or in combination with other current chemotherapies or immunotherapies (anti-PD1 or anti-CTLA4) as well as new vaccines.

Among vaccine formats, recent clinical studies using ivt mRNA for cancer treatment showed very promising results (Sahin et al., 2017a). Both ivt mRNA coding shared tumor antigen or individual mutations can be used to prime/restimulate anti-cancer specific immunity (Kreiter et al., 2015; Rittig et al., 2011; Sahin et al., 2017a). Functionality of ivt mRNA has been very much increased through utilization of stabilization sequences (5′ and 3′ UTRs) (Carralot et al., 2004; Holtkamp et al., 2006; Kariko et al., 1999), optimal 5′ cap (Kuhn et al., 2011), optimized open reading frame (codon usage) (Gustafsson et al., 2004; Malarkannan et al., 1999) and extended poly A tail (Holtkamp et al., 2006). Still, further optimization of ivt mRNA functionality can be envisioned based on the utilization of modified nucleotides or incorporation of helper sequences. Concerning the first aspect, understanding the role of RNA modifications (see above) can be used to generate better ivt mRNA. Investigating the second aspect, we could show that employing an aptamer that attracts the eIF4G protein at the 5′ end of the ivt mRNA can enhance translation.

Integrating epitanscriptomics, improved ivt mRNA-based formulations and individual parameters (identification of mutations, immune fitness) we aim at defining personalized immunochemotherapy treatments that will efficaciously and safely cure or prevent cancers.

8. Literature

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9. Curriculum vitae

Curriculum Vitae

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